The role of the cell adhesion molecule NCAM and the transcription factor Dlx2 in epithelial-mesenchymal transition (EMT) and tumor progression

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SUMMARY

Cancer belongs to the most life-threatening diseases in humans and represents in a simplified manner the destruction of healthy tissue and organs by uncontrolled cell proliferation and subsequent formation of a tumor. The most dangerous step of this disease occurs when cancer cells gain the ability to invade into the surrounding tissue and to disseminate via the blood system or the lymphatics throughout the body to form at distant sites secondary tumors, a process named metastasis. To gain motility and invasiveness, cancer cells are known to undergo an epithelial-mesenchymal transition (EMT). EMT is well known from non-pathological processes like development and wound healing and describes the temporal transition of non-invasive epithelial cells into motile, invasive mesenchymal cells.

To gain new and more detailed insights into the complex process of EMT and to identify new potential markers for ongoing metastasis, we established different *in vitro* EMT model systems and tracked changes in global gene expression occurring during EMT. By comparing these gene expression profiles we identified the neural cell adhesion molecule (NCAM) and the homeobox transcription factor distal-less homeobox 2 (Dlx2) to be upregulated during EMT.

Employing different *in vitro* systems such as the normal murine mammary gland (NMuMG) cells which undergo a progressive EMT upon transforming growth factor (TGFβ) treatment, in combination with transgenic and syngeneic mouse models, we investigated the role of NCAM and Dlx2 during the process of EMT.

Our investigations revealed that NCAM expression is required and sufficient to induce EMT in NMuMG cells. We show that during EMT NCAM undergoes a functional switch by changing both its subcellular localization and its interactions partners. A subset of upregulated NCAM breaks down its complex formation with the fibroblast growth factor receptor (FGFR) and translocates into lipid rafts where it interacts with the member of the Src family kinase (SFK) $p59^{Fyn}$. In association with $p59^{Fyn}$ NCAM induces the phosphorylation of focal adhesion kinase (FAK), leading to stabilization of β1-integrin-mediated focal adhesion, increased cell spreading and migration. In line with this observation, we found NCAM

expression at the invasive front of human and murine tumors.

In contrast, Dlx2 function is not required and its expression is not sufficient to induce EMT in NMuMG cells. Instead, we found that Dlx2 function protects from TGFβ-induced cell-cycle arrest and apoptosis by two major modifications namely, inhibition of the apoptotic, canonical TGFβ-signaling pathway and the activation of mitogenic, survival-ensuring mitogen-activated protein kinase (MAPK) -and phosphoinositide 3-kinase (PI3K) pathways. The canonical apoptotic TGFβ signaling is inhibited by transcriptional repression of the TGFβ receptor II (TGFβRII) gene, leading to reduced TGFβRII protein levels, decreased activation of the signal transducers Smad2/4 and reduced transcriptional activation of the cell-cycle inhibitors such as $p21^{CIP1}$. Proliferation and survival is mediated by the cooperated activation of the MAPK and PI3K pathways triggered by epidermal growth factor receptor (EGFR). Supporting the importance of Dlx2 function during tumor development and progression, we show that (i) loss of Dlx2 function in B16 melanoma cells significantly impairs their ability to form primary tumors and metastatic lesion in the lung of transplanted syngeneic mice and (ii) expression of Dlx2 correlates significantly with invasiveness of human melanoma, lung and prostate cancers.

In summary, we identified the cell-adhesion molecule NCAM and the transcription factor Dlx2 as important key players of EMT by promoting invasion and survival, respectively. Whether these genes can be used as prognostic markers for EMT-driven tumor invasion requires further investigations.

ZUSAMMENFASSUNG

Krebs gehört zu den gefährlichsten Krankheiten des Menschen. Krebs sind entartete Zellen, die durch unkontrolliertes Wachstum und Vermehrung gesundes Gewebe und Organe zerstören. Dabei erfolgt der lebensbedrohlichste Schritt dieser Krankheit, wenn Krebszellen sich im Gewebe ausbreiten, invasiv werden und durch das Blut- und Lymphsystem in verschiedenen Körperregionen transportiert werden, um dort zu sekundären Tumoren auszuwachsen. Dieser Prozess wird als Metastasenbildung bezeichnet.

Damit Krebszellen mobil und invasiv werden können, vollziehen sie ein sogenanntes *Epithelial-Mesenchymal Transition* (EMT). EMT ist ein Vorgang, der bei nicht-pathologischen Prozessen wie z.B. der Embryonalentwicklung oder Wundheilung stattfindet und die temporäre Umwandlung nicht-invasiver Epithelzellen in invasive, fibroblastoide Zellen beschreibt.

Um neue und tiefere Einblicke in die komplexen Prozesse des EMT zu bekommen und um neue potentielle Marker für die Metastasenbildung identifizieren zu können, haben wir verschiedene *in vitro* EMT-Modelle etabliert und die Veränderungen der Genexpression, die während EMT stattfinden, verfolgt. Durch das Vergleichen dieser Genexpressionsprofile konnten wir unter anderem das neuronale Zelladhesionsmolekül NCAM und den Transkriptionsfaktor *distal-less homeobox 2* (Dlx2) als in EMT erhöht exprimierte Gene identifizieren.

Unsere Untersuchungen haben ergeben, dass die Funktion von NCAM für EMT benötigt wird. Darüber hinaus reicht seine alleinige Überexpression aus, um EMT auszulösen. Wir zeigten, dass NCAM während EMT eine funktionelle Wandlung durchmacht, indem es sowohl seine subzelluläre Lokalisation als auch seine molekularen Interaktionspartner wechselt. Ein Teil des hochregulierten NCAM löst sich von seiner Interaktion mit dem Fibroblasten Wachstumsfaktor-Rezeptor (FGFR) und wandert in sogenannte "lipid rafts", wo es mit der Src-Kinase $p59^{Fyn}$ interagiert. In Kooperation mit $p59^{Fyn}$ induziert NCAM die Phosphorylierung der Fokalen Adhäsions-Kinase (FAK), wodurch β1-integrin vermittelte, fokale Adhäsion stabilisiert und somit Zelladhäsion und Zellmigration verstärkt wird. In

Übereinstimmung mit diesen Ergebnissen konnten wir die Lokalisation von NCAM in der invasiven Front von Tumoren bei Mensch und Maus zeigen.

Im Gegensatz zu NCAM wird die Funktion von Dlx2 für EMT nicht benötigt und ist nicht ausreichend, um EMT auszulösen. Stattdessen schützt Dlx2 vor dem transformierenden Wachstumsfaktor beta (TGFβ) vermittelten Zellzyklusarrest und Zelltod. Dlx2 ist in der Lage sowohl die durch TGFβ ausgelöste Signalkaskade als auch die Mitogen-aktivierte Protein-Kinase (MAPK) und Phosphoinositide-3 Kinase (PI3K) Signaltransduktionkaskaden zu verändern. Die durch TGFβ induzierte apoptotische Signaltransduktion wird durch die transkriptionelle Repression des TGFβ-Rezeptors II (TGFβRII) inhibiert, wodurch dieser die Signalüberträger Smad2/4 nicht mehr aktivieren kann, was wiederum zu einer verminderten Expression des Zellzyklusinhibitors $p21^{CIP1}$ führt. Komplementär wird die Teilungsfähigkeit und das Überleben der Zelle durch die kooperative Aktivität der MAPK and PI3K ermöglicht, die durch den Epidermalen Wachstumsfaktor-Rezeptor (EGFR) kontrolliert werden. Die Wichtigkeit der Dlx2 Funktion während der Tumorentwicklung und -progression wir durch folgende Beobachtungen gestützt: (i) Der Verlust der Dlx2 Funktion schränkt die Fähigkeit von B16 Melanomzellen sowohl Tumore als auch Lungenmetastasen zu bilden signifikant ein und (ii) die Expression von Dlx2 korreliert signifikant mit der Aggressivität von humanem Prostatakrebs und Melanomen.

Somit konnten wir zeigen, dass sowohl das Zelladhäsionsprotein NCAM als auch der Transkriptionsfaktor Dlx2 eine wichtige regulatorische Rolle während EMT spielen. Dabei ist NCAM entscheidend für die Ausbreitung der Krebszellen in umliegendes Gewebe, während Dlx2 das Wachstum und Überleben der Zellen in Gegenwart von TGFβ ermöglicht. Ob NCAM und/oder Dlx2 als diagnostische Marker für Tumorprogression und Metastasenbildung im humanem Krebs genutzt werden können, bedarf weiterer Untersuchungen.

1. Introduction

1.1. Hallmarks of cancer

Cancer represents in a simplified manner the destruction of healthy tissue and organs by uncontrolled cell proliferation and subsequent formation of a tumor. Multiple intrinsic (e.g. reactive oxygen species) as well extrinsic factors (e.g. UV light) can provoke cancer formation by inducing DNA damages leading to inactivation of tumor-suppressors or the arise of oncogenes. Cells which transform into a cancer cell have to acquire distinct "hallmarks of cancer" to grow out into a tumor (Hanahan and Weinberg, 2000):

- Self-sufficiency in growth signals. Growth factors which are required for proliferation are limited and have to be supplied by external sources. Cancer cells break down this limitation by either producing growth factors in an autocrine manner and/or by overexpressing growth factor receptors to become hyperresponsive towards ambient growth factor concentrations (Fedi et al., 1997).
- Insensitivity to antigrowth signals. Within normal tissue, multiple anti-proliferative signals operate to maintain cellular quiescence and tissue homeostasis. Tumor cells block antigrowth signaling by inactivating cell-cycle inhibiting pathways such as the retinoblastoma (pRb) pathway or by overexpressing mitogenic proteins such as c-Myc (Weinberg et al., 1995).
- Resistance towards apoptosis. Several intrinsic factors (e.g. genomic instability) as well as extrinsic factors (e.g. hypoxia) induce apoptosis of untransformed cells. Tumor cells prevent apoptosis by overexpressing anti-apoptotic genes such as Bcl-2 or by silencing tumor-suppressors such as p53 (Butt et al., 1999, Harris et al., 1996).
- Limitless replicative potential. Untransformed cells are limited in their replicative potential by the length of their telomeres. Shortening of telomeres below a certain length induces cell-cycle arrest, senescence and apoptosis. Tumor cells prevent this by expressing the telomerase enzyme (Shay and Bacchetti et al., 1997)

- Sustained angiogenesis. The growth of untransformed cells is limited by nutrients and oxygens supplied by blood vessels. Tumor cells bypass this limitation by inducing angiogenesis via release of angiogenic factors such as vascular endothelial growth factor (VEGF) and/or fibroblast growth factor (FGF) (Folkman et al., 1997).
- Tissue invasion and metastasis. Non-transformed epithelial cells are embedded via tight intercellular junctions into a tissue, which is limiting in space and nutrient supply. Tumor cells break down this limitation by gaining invasiveness and the ability to metastasize to distant sites (Sporn et al., 1996). For more details see Chapter 1.2 "Distinct mechanisms of tumor invasion and metastasis" and Chapter 1.4 "EMT, the cytoskeleton, and cancer cell invasion.

1.2. Distinct mechanisms of tumor invasion and metastasis (Review)

Mahmut Yilmaz, Gerhard Christofori, and Francois Lehembre: "Distinct mechanisms of tumor invasion and metastasis" Trends in Molecular Medicine 2007, 13(12), 535-41

Purpose of the review

Cancer cells utilize different types of migration to leave the primary tumor in order to reach distant sites within the body. This review highlights the characteristics of each type of migration and summarizes the current knowledge about the strategies used by invasive cancer cells to establish secondary tumors at distant sites.

Summary

Cancer cells are able to invade into the surrounding tissue utilizing different types of migration namely (i) collective migration, including coordinated as well as cohort migration and (ii) single cell migration, including mesenchymal as well as amoeboid migration. Due to their intrinsic plasticity, cancer cells are able to switch between different modes of migration and thus are able to adapt to changes in their environment.

1.2.1. Introduction

Metastasis is the cause of 90% of all deaths from cancer and it displays a remarkably diverse set of clinical features. In principle, metastatic secondary tumors are formed by cancer cells that have left the primary tumor mass and traveled, mainly via blood and lymphatic vessels, to seek out new sites throughout the body where they seed new colonies. To achieve this journey, cancer cells employ numerous strategies that all lead to the same goal, the establishment of secondary sites. In order to leave the primary tumor and to disseminate to distant organs, metastatic cancer cells first lose adhesion to neighboring tumor cells and gain migratory and invasive capabilities. Such epithelial-mesenchymal-transition (EMT) is accompanied by a variety of changes in gene expression and functions, such as the loss of epithelial markers and the gain of mesenchymal markers (Thiery and Sleeman, 2006). Cancer cells are then able to permeate the basement membrane, to invade into surrounding tissue and to gain direct access to blood and lymphatic vessels. After successful intravasation into

vessels they need to survive in the blood or lymphatic circulation, to disseminate in the whole body to then recognize specific target organs and extravasate into a new environment where they seed and grow as secondary tumors. The ability to grow in secondary sites is of particular importance, since the 'foreign' tissue environment does not necessarily provide cancer cells with the familiar collection of growth and survival factors that allowed them to thrive in the primary 'home' tumor. Depending on the features of the primary tumors, their stroma and the intrinsic ability of a given metastatic cell to adapt to a new stroma, cancer cells will use distinct mechanisms to proliferate, survive and spread. Thus, the multiple steps of metastases encompass many biological functions. To achieve these functions, cancer cells frequently hijack gene expression programs employed by non-transformed cells during embryonic development, such as EMT and a variety of signaling pathways underlying morphogenetic processes.

1.2.2. Cancer cells use multiple strategies of invasion

Depending on the tumor type and the surrounding tissue, cell migration involves different cellular strategies to overcome the physical resistance of three-dimensional tissue networks (Figure 1). Accordingly, various patterns of invasion can be observed upon morphological analyses of different human cancer types (Friedl, 2004). For example, squamous cell esophageal cancers predominantly invade by forming cone-like structures, lobular breast cancers migrate in indian-file patterns through the extra-cellular matrix (ECM), and anaplastic thyroid cancers mainly show invasion of single, sparse cells. Based on histological criteria, there are two fundamentally different patterns of invasion: single cell invasion and collective cell invasion. On histological sections, single cell migration is characterized by the presence of isolated and dispersed tumor cells in an adjacent tissue. This is in contrast to what is observed during collective cell invasion where the cancerous tissue pushes forward as a whole, thereby displacing the healthy surrounding cells.

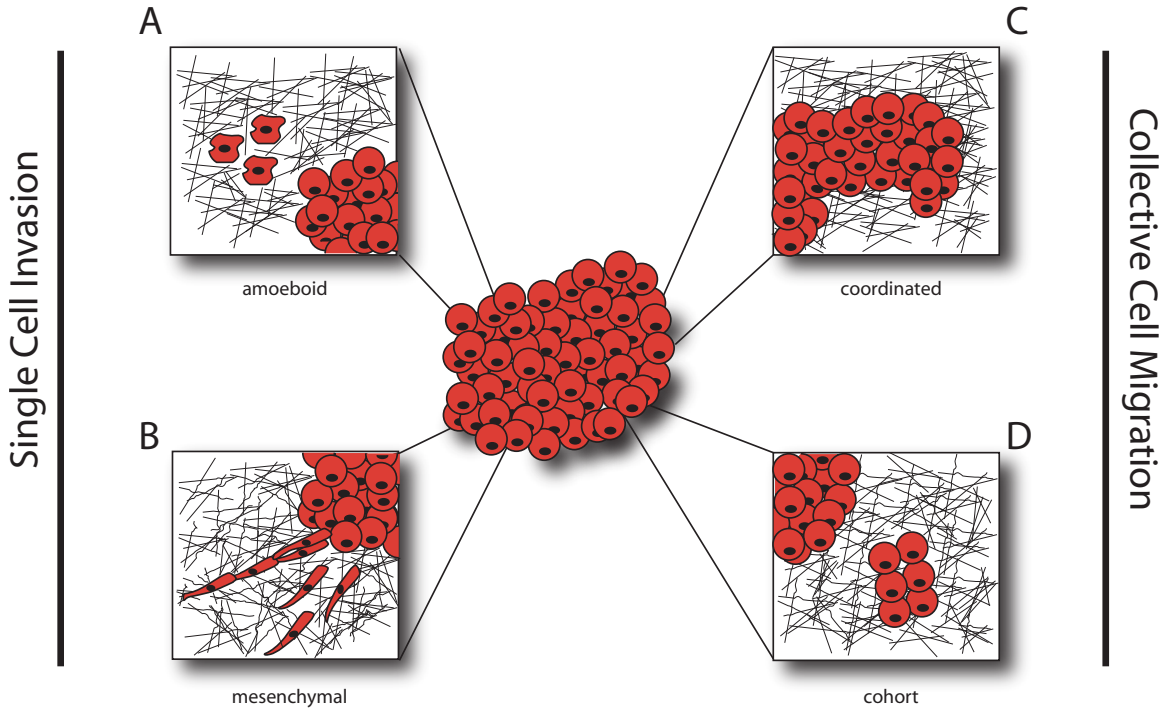


Figure 1: Different strategies of tumor invasion. (A) Individual cancer cells detach from the primary tumor and invade the surrounding tissue by amoeboid invasion. Characteristics for the amoeboid invasion are the weak interactions with the ECM and the protease -and calpain independency. **(B)** The mesenchymal invasion is initiated by an epithelial-mesenchymal transition (EMT) of individual cancer cells at the invading front of the primary tumor. Characteristics for the mesenchymal invasion are the spindle shaped morphology of the cancer cells and the expression of proteases and certain set of integrins. Its is also assumed that the invading cancer cells are able to switch between both the amoeboid and mesenchymal invasion depending on the composition of the microenvironment they meet. **(C)** In the coordinated collective invasion cancer cells invade the surrounding tissue without loosing the contact to their neighbour cells and the primary tumor. This type of invasion is protease dependent. **(D)** The collective invasion as a cohort of 6-10 cells is characterized by the remaining contact between the invading cells and the lost contact to the primary tumor. This type of invasion is protease dependent.

1.2.3. Single migrating cells

Cancer cells migrating and invading as single cells can either employ a fibroblast- or a leukocyte-like strategy to passage through the ECM:

1.2.3.1. The fibroblast strategy: mesenchymal migration

When individual malignant cells detached from the primary tumor mass at the periphery of carcinomas they frequently display many hallmarks of EMT (Brabletz et al., 2005b). The

essential features of EMT are the disruption of intercellular contacts, the acquisition of a spindle-shape fibroblast-like morphology, the enhancement of cell invasiveness and cell-stroma interaction and a slow division rate (Lee et al., 2006). Altogether, these features lead to the release of "mesenchymal" cells from the parent epithelial tissue. The resulting mesenchymal-like phenotype is amenable to migration and, by extension, to tumor invasion and metastatic dissemination. Interestingly, a typical feature of some cancers might correspond to an *in vivo* EMT: neural crest tumor and also melanoma cells migrate one-by-one in single Indian-files through channels they have carved in the adjacent stroma (Friedl, 2004). These invading cells often lose E-cadherin expression and show several characteristics of active invasion, including expression of integrins and surface proteases (Figure 1).

While numerous markers of mesenchymal cells, such as vimentin, N-cadherin or fibronectin are readily identified in cells undergoing EMT *in vitro*, very few of these markers are detected in invading cells in cancer biopsies *in vivo*, except the loss of E-cadherin expression and the gain of a nuclear β-catenin signal (Brabletz et al., 2005a). Moreover, *in vivo*, epithelial markers are frequently maintained in invading cancer cells, including the expression of cytokeratins that are routinely used for cancer diagnosis. The failure of readily detecting full EMT in cancers of patients has lead to a debate whether EMT actually occurs in patient carcinogenesis (Tarin et al., 2005; Thompson et al., 2005). However, since the loss of E-cadherin expression is observed in almost all epithelial cancers and other morphological and genetic hallmarks of EMT can be detected with varying frequency, major efforts are ongoing to dissect the molecular pathways underlying the transition from an epithelial to a mesenchymal phenotype during tumor progression (Christofori and Semb, 1999). Profiling gene expression of primary cancers and metastasis as well as of cancer cells undergoing EMT will certainly help to settle this issue and to identify novel markers of tumor malignancy.

1.2.3.2. The leukocyte strategy: amoeboid migration

Amoeboid migration is a very efficient invading mode where tumor cells use a crawling type of movement that is driven by weak interactions with the stroma (Hlubek et al., 2007). Such highly deformable cells have been observed *in vivo* using intravital imaging of carcinoma cells (Sahai, 2005). Proteases are not required for this movement because cells are able to

squeeze through gaps in the ECM (Figure 1). Accordingly, amoeboid migration was recently described by its extracellular proteases- and calpain-independent mechanism of action (Wolf et al., 2003a; Sahai et al., 2007). This type of migration is retained in leukocytes and certain cancer cells, including hematological cancers and neuro-endocrine tumors, such as small-cell lung cancer (Friedl, 2004).

1.2.4. Collectively migrating cells

A hallmark of collective invasion is the maintenance of cell-cell adhesion by the continuous expression of adhesion molecules and other components of the adhesive apparatus by invading cells. A subset of highly motile cells at the invading front of the cell sheet (referred to as path-generating cells) provides the migratory traction and via cell-cell junctions pulls the following cells in their rear (Carragher et al., 2006). Collectively migrating cells engage and cluster integrins in anterior protrusions towards the ECM and show a high expression of specific matrix-metalloproteases (MT1-MMP, uPA/uPAR, MMP-2) for remodeling of the ECM (Hegerfeldt et al., 2002). The molecular mechanisms involved in collective cell invasion are likely to resemble that of morphogenetic collective cell movements observed during embryonic development.

1.2.4.1. The morphogenesis strategy: coordinated invasion

Coordinated invasion consists of cell sheets that extend into tissue, yet maintain contact with the primary tumor. Cells present at the migrating front produce MMPs that generate a path for the cells to follow. This type of movement is observed in invasive epithelial cancer such as oral squamous cell carcinoma, basal cell carcinoma, melanoma, mammary carcinoma and colon carcinoma (Nabeshima et al., 2000). This mass movement is similar to what is observed in embryonic morphogenesis, where complex multi-cellular sheets formed by cells that are linked to each other by cell-cell junctions move along surrounding tissue substrates (Figure 1). The precise molecular mechanisms underlying this coordinated invasion are still largely unknown, mainly due to the difficulties to model this complex type of migration *in vitro*. Our laboratory has recently reported that the cells in the leading front of collectively invading squamous cell carcinomas express high levels of podoplanin, a transmembrane

glycoprotein implicated in the remodeling of the actin cytoskeleton (Friedl et al., 2004). Notably, when expressed in epithelial breast cancer cells, podoplanin is able to induce filopodia formation and cell migration and invasion without the downregulation of E-cadherin expression, indicating a mode of cell migration that clearly does not involve EMT (Wicki et al., 2006).

1.2.4.2. The border-cell strategy: cohort migration

This specific pattern of migration and invasion is manifested by cell-clusters that collectively detach from the primary tumor to generate migrating cell nests. During this process, cells continue to adhere to each other by adherens junctions and leave behind large gaps in the matrix, indicating that they are able to degrade the ECM (Figure 1). Leading edge cells express integrins, which enable their attachment to the ECM ahead of the cell nests. This type of invasion is frequently found in a subset of melanomas and in rhabdomyosarcomas (Nabeshima et al., 2000). This phenomenon is also observed in primordial germ cell migration and in Drosophila border-cell migration, where a group of 6–10 cells specified at the anterior of the egg chamber migrate towards the developing oocyte (Wicki and Christofori, 2007). That the genes playing a key role during primordial germ cell and border-cell migration are also regulating cohort migration in human cancers is an attractive thought, yet remains to be determined.

1.2.5. Tumor cell plasticity

Several lines of evidence suggest that tumor cells exhibit high plasticity in modulating their type of invasion in order to progress through the multiple stages of the metastatic process. For example, to intravasate collectively invading cells have to break away from their neoplastic neighbors before being able to enter the blood stream. Thus, an adaptive switch in migration mechanisms must occur, allowing cells to survive in different environments. Supporting this hypothesis, cells not only undergo EMT during tumor metastasis but also progress from a mesenchymal to an amoeboid type of invasion. For example, mesenchymal cells subjected to protease inhibitor treatment in 3-D culture undergo a mesenchymal-

amoeboid transition, allowing efficient migration in the absence of protease activity (Sahai et al., 2007). Friedl and co-workers have also shown in a melanoma explant model of human cancer that cells can switch from one invasion pattern to another, depending on the tumor environment and particularly on the selective pressure exerted by cancer drugs (Carragher et al., 2006). In addition, adherens junction components, such as E-cadherin, are known to be lost in primary tumors but are re-expressed in distant metastases suggesting that mesenchymal cells can re-differentiate into an epithelial phenotype upon reaching sites of metastases (Brabletz et al., 2005b). These experimental data suggest a highly efficient cellular and molecular plasticity in tumor cell migration strategies (Montell, 2003).

1.2.6. Acid-mediated tumor invasion

The vast majority of cancers exhibit increased glucose flux compared to normal tissue (Friedl and Wolf, 2003a). This glycolytic phenotype plays a critical role for the survival of tumor cells exposed to a hypoxic environment (Gambhir, 2002). Interestingly, it has been proposed that elevated glycolysis and with it higher acid production leads to cell necrosis and apoptosis. Cell death produces gaps and spaces into which migratory cancer cells could invade. In addition, necrotic cells release proteolytic enzymes that further support extracellular matrix degradation (Gatenby and Gillies, 2004).

1.2.7. Establishment of secondary sites

In most cancers, metastases are seeded via either hematogenic or lymphogenic dissemination. It is estimated that several million cells per gram of tumor can be shed daily into the lymphatic system or in the bloodstream (Pennacchietti et al., 2003; Gatenby et al., 2006). However, the actual process of cancer cell intravasation is only poorly understood. Intravasation into blood vessels may be an active phenomenon where tumor cells migrate towards nutrient or chemokine gradients or a passive phenomenon that does not involved active cancer cell migration but rather simple cancer cell shedding into the highly permeable tumor vasculature (Liotta et al., 1976). A potential mechanism of chemoattraction has been recently suggested: tumor cells can secrete both CCR7 receptor and its ligands, CCL21 and CCL19. The ligands are then drained towards lymphatics by the flow of tissue fluid and

concentrate downstream of cancer cells ensuring that they migrate towards lymphatic vessels (Butler and Gullino, 1975). The dissemination of tumor cells has characteristic patterns of organ tropism that reflects their heterogeneity and depends on the cancer type (Bockhorn et al., 2007). While the circulatory pattern can explain the location of certain metastases, it is not sufficient to explain the incidence of metastases in most secondary organs(Shields et al., 2007). More than a hundred years ago, Paget proposed that disseminating tumor cells (seed) need to find the appropriate microenvironment in distant organs (soil) for metastatic outgrowth ('seed and soil' hypothesis; (Nguyen and Massague, 2007)). More recently, cytokines have been shown to play crucial roles in the homing of tumor cells to distinct organs. For example, the chemokine-receptor CXCR4 is highly expressed in malignant breast cancers and their metastases. Organs representing the first destinations of breast cancer metastases express high levels of SDF1/CXCL12, the bona fide ligand of CXCR4 receptor, suggesting an attraction of the receptor-expressing tumor cells toward an SDF1/CXCL12 gradient. Neutralizing the interactions between SDF1/CXCL12 and CXCR4 in a transgenic mouse model *in vivo* significantly impairs metastases of breast cancer cells to regional lymph nodes and lung (Fidler, 2003). Thus, specific expression of a set of genes may direct metastases to distinct secondary sites. Accordingly, in examining breast cancer cell with varying propensity to metastasize to different target organs, specific gene expression signatures have been identified, for example a lung-metastasis signature and a bone metastasis signature (Paget, 1889; Muller et al., 2001) (see also below). In addition to seeding de novo metastasis, invasive tumor cells may also contribute to primary tumor growth: disseminated cells may return to their original site leading to progressive accumulation of very aggressive cells in the primary tumor and thus local recurrence, a self-seeding hypothesis that is support by a number of experimental and clinical observations (Minn et al., 2005).

1.2.8. Circulating tumor cell extravasation

Before establishing metastases, cancer cells need to extravasate from the blood stream. To do

so, cancer cells adhere to the vascular endothelium, transmigrate across the endothelial cells and invade the surrounding tissue. The molecular mechanisms underlying these processes are only poorly understood. Besides specific adhesion of cancer cells to surface receptors on endothelial cells, platelets have been proposed to facilitate this process through several mechanisms. Actually, Armand Trousseau proposed already in 1865 that a visceral carcinoma could cause thrombophlebitis, an inflammation of the vessels due to blood clotting (Trousseau's sign) (Kang et al., 2003). Circulating cancer cells may use platelets as shields in order to promote their survival (Norton and Massague, 2006). Platelets also protect tumor cells from the cytotoxic effects of circulating TNFα, and enhance tumor embolization in the microvasculature. Furthermore, platelets facilitate cancer cell adhesion to the endothelium and are able to induce neoangiogenesis enabling growth in the secondary site (Philippe et al., 1993; Nieswandt et al., 1999; Kang et al., 2003; Gupta and Massague, 2004). In a transgenic mouse model of liver carcinogenesis, caused by the expression of the c-MET oncogene, venous thromboses (Trousseau's sign) develop apparently dependent on the upregulation of plasminogen activator inhibitor type 1 (PAI-1) and cyclooxygenase-2 (COX-2) (Mehta, 1984). Indeed, administration of aspirin to mice reduces the metastatic potential of cancer cells, presumably both by reducing the incidence of cancerous emboli and the $\alpha_V\beta_3$ integrin-mediated angiogenesis (Karpatkin and Pearlstein, 1981; Nash et al., 2002; Boccaccio et al., 2005). Colonization of a secondary organ by disseminated cancer cells, however, may not only be achieved by embolization in a capillary field, but also by adhesive interactions between surface receptors and ligands on cancer and endothelial cells. For example, $\alpha_3\beta_1$ integrins and a novel receptor called metadherin are implicated in homing to the microvasculature of the lung (Gasic et al., 1972; Dormond et al., 2001). For the actual extravasation process, both the motility of the cancer cells and the permeability of the vascular endothelium are important. Ablation of the cytoskeletal adaptor protein ezrin in osteosarcoma cells reduces the escape of cancer cells from the vasculature (Wang et al., 2004). The angiogeneic factor VEGF, highly expressed by most cancer cells, increases the permeability of the endothelium and thus facilitates extravasation (Brown and Ruoslahti, 2004). Therefore, several pathway may be involved alone or in combination in cancer cell

extravasation, yet the molecular details remain to be elucidated. Recently, Massagué and coworkers reported that four genes (epiregulin, COX2, MMP-1 and MMP-2) collectively contribute to vascular remodeling functions by supporting the formation of vasculature in mammary tumors, the entry of tumor cells into the circulation and the exit of tumor cells from the bloodstream into the lung parenchyma. When any of these genes is singly inactivated in breast cancer cells, either by pharmacological inhibition or by RNAi-mediated knock-down technology, there is only a moderate inhibition of primary-tumor growth and lung metastasis. However, when all four genes together are inactivated, an almost complete abrogation of both primary-tumor growth and lung metastasis is observed (Khanna et al., 2004).

1.2.9. Establishment of a pre-metastatic niche

Another attractive hypothesis to explain how cancer cells recognize and colonize specific organs is the generation of a permissive microenvironment in the metastatic target organ for incoming cancer cells (pre-metastatic niche) (Weis and Cheresh, 2005). Recently, employing syngeneic tumor transplantation models of organ-specific metastasis, it has been shown that bone-marrow-derived, VEGF receptor-1-expressing progenitor cells first home to pre-metastatic sites in target organs before the arrival of tumor cells. One of the cues for incoming cancer cells seems to be an increase in the deposition of fibronectin (Gupta et al., 2007). However, the actual signals that lead to the tissue-specific recruitment of progenitor cells are unknown. Another study has shown that the release of VEGF, TGFβ and TNFα by primary tumors induces expression of the chemoattractants S100A8 and S100A9 by lung endothelium and myeloid cells which in turn facilitates the colonization of pre-metastatic sites within the lung parenchyma by cancer cells (Kaplan et al., 2007).

1.2.10. Are cancer initiating cells seeds for metastasis?

The morphological resemblance of the metastatic lesions to the primary tumor and the presence of metastatic poor prognosis gene expression pattern in primary tumors led to the hypothesis that the development of metastatic cancer cells is rather an early than a late event during tumor progression (Ramaswamy et al., 2003; Kaplan et al., 2005; Hiratsuka et al.,

2006). In the last years, cancer stem cells (CSC) or better cancer initiating cells (CIC) have been suggested to play a critical role during the metastatic process (Sorlie et al., 2001). CIC are defined as cancer cells that are able to self-renew and to initiate tumor formation. Since metastases seem to recapitulate the heterogeneous phenotype of their primary tumor, it has been suggested that CICs escaping from the primary tumor could be the seed for metastasis (van 't Veer et al., 2002). This notion is supported by the observation that only a subset of the cancer cells from a primary tumor or from a metastatic site can induce new metastatic lesions (clonal event). In addition, the pathological features of metastatic cancer cells resembles in great parts characteristics of bona fide stem cells (Table I). At the moment there are different models attempting to explain the origin and nature of CIC ((Sorlie et al., 2001); Figure 2): (i) CIC derive from normal stem cells that upon oncogenic transformation accumulate mutations over time, due to their self-renewal capacity, (ii) transient amplifying progenitor cells which are more abundant and retain partial self-renewal capacity are transformed and acquire additional mutations over time, (iii) fusion of stem cells with transformed cells gives rise to transformed cells with self-renewal capacity, which could explain the detection of fusogenic markers (e.g. CD44 or CD47) and aneuploidy in neoplastic transformation, (iv) normal cancer cells regain self-renewal capacity and de-differentiate due to specific mutations or environmental cues (stem cell niche). Therefore, the term 'cancer initiating cell' probably relates to a broad group of cells that share some common properties. Identification of these cells in tumors and metastases will provide novel insights into tumor biology in general and into the metastatic process in particular.

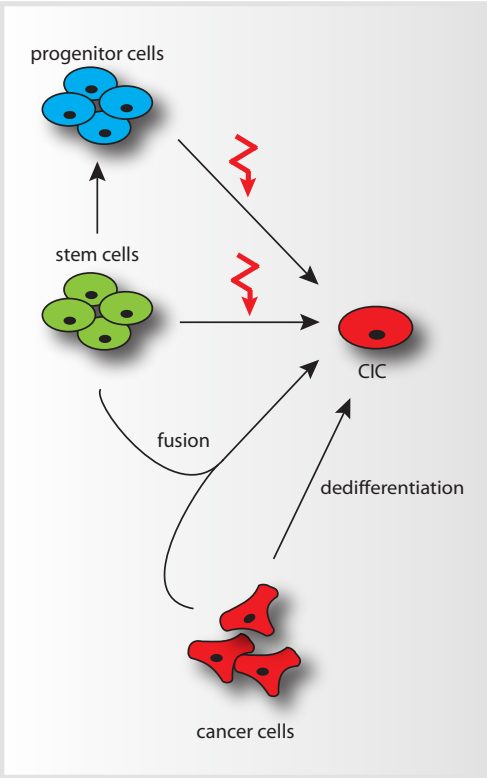


Figure 2: Different models for the development of a cancer initiating cell (CIC). (A) The inappropriate fusion of a normal cell and tumorigenic cell can give rise to a CIC. Fusion can occur either between a normal stem cell and a cancer cell or between a mutated stem cell and a normal cell. Both scenarios lead to a hybrid CIC, which unites features of both fusogenic partners and could mark the onset of tumor development and metastasis. A third, more unlikely scenario could be the fusion of both a mutated stem cell and a cancer cell. (B) Single or multiple mutations within a differentiated cell, immediate progenitor cell or stem cell could give rise to a CIC.

1.2.11. Relevance for patients

The risk of metastatic spread and/or recurrence can sometimes be predicted from certain features of the primary tumor, as in the case of breast cancer, for which tumor size, histological grade, and gene expression pattern are indicative of the risk of relapse (Goldhirsch et al., 2006). However, these approaches are still limited in their prognostic value, and we need to learn more about the molecular mechanisms underlying the multiple stages of tumor metastasis in order to substantially improve the cancer patients' condition. Recent studies showed that 70 to 80% of breast cancer patients receiving adjuvant treatment

would have survived without it. These patients currently are not identified, and their treatment protocols may be inappropriate(Group., 1998a; Group., 1998b). The failure to distinguish bona fide metastases from inert disseminated cells thus has important implications. It seems important to develop novel surrogate markers of tumor progression that may identify the state of the metastatic process in cancer patients. Moreover, depending on the invasion program used by cancer cells, molecular mechanism-based therapies should be developed. Hence, morphological and molecular features of the invasive tumor front are important for therapeutic considerations, not only for choosing the right therapeutic approach but also for its pharmacological characteristics. For example, collective invasion coincides with intact cell junctions and thus higher cancer cell density, which in turn affects the penetration of drugs into solid tumors (Minchinton and Tannock, 2006). Moreover, patient diagnosis based on certified molecular markers of distinct tumor invasion or metastatic programs could go along with the use of specific drugs in a more accurate way (Sotiriou and Piccart, 2007). In the near future, gene expression analysis will be routinely employed for deciphering a patient's prognosis. For example, in breast cancer, several existing gene expression signatures have been shown to be equally informative, and it has been suggested that combining them might be even more powerful(Massague, 2007). Obviously, the main challenge in the future will be the development of drugs (and/or combinatorial treatment) that not only target rapidly dividing but also slowly dividing, highly malignant cancer cells. The molecular understanding of the early steps of cancer cell invasion will thus provide novel therapeutic opportunities.

1.2.12. Concluding remarks

It is now well established that various cancer types use distinct and complex strategies to disseminate in the whole body. Future studies of these mechanisms will involve the establishment of robust *in vitro* 3-dimensional invasion systems. This challenging endeavor needs to consider that depending on the tumor location the tumor stroma has different characteristics. For example, malignant breast cancer cells can revert to a non-transformed phenotype when exposed to a non-permissive stroma(Albini and Sporn, 2007). Moreover, irradiated tissue can induce non-transformed cells to acquire malignant potential. Models

combining *in vitro* cell culture experiments together with *in vivo* imaging in appropriate mouse models will be one of the solutions to address this challenge (Wyckoff et al., 2007).

1.3. TGFβ - its role in tissue homeostasis and cancer cell invasion

1.3.1. The two faces of TGFβ

TGFβ plays a central role in various biological processes including development, tissue homeostasis and cancer. During developmental processes, such as gastrulation or neural crest formation, TGFβ induces motility and invasiveness, enabling cells to migrate to distant sites within the developing body. In contrast, in differentiated epithelial tissue TGFβ is mainly known to maintain tissue homeostasis by inducing growth arrest and apoptosis, thus functioning as a tumor-suppressor (Derynck et al., 2001; Siegel and Massague, 2003; Massague, 2008). This complex and ambivalent nature of TGFβ signaling plays also a critical role in cancer development and progression. Here, TGFβ acts at early stages of tumorigenesis as a tumor-suppressor by inducing cell cycle arrest and apoptosis. During later stages of tumorigenesis, TGFβ functions as an oncogene by inducing the production of mitogenic factors, evasion of immunity and the transition of non-invasive epithelial cells into invasive mesenchymal cells, a process named epithelial-mesenchymal transition (EMT) (Roberts and Wakefield, 2003; Thiery and Sleeman, 2006; Pardali and Moustakas, 2007; Massague, 2008; Yang and Weinberg, 2008). Cancer cells which have undergone EMT are able to detach from the primary tumor and to invade into the surrounding tissue to form metastatic lesions at distant sites.

1.3.2. TGFβ signaling

TGFβ is the prototype of the TGFβ superfamily of morphogenetic factors, including bone morphogenetic protein (BMPs), growth differentiation factors (GDFs), Müllerian Inhibiting Substance (MIS), activins and others. All these cytokines are well known to regulate various biological processes like cell proliferation, apoptosis, differentiation, angiogenesis and migration. Each ligand presents unique features of action, while they all share a common machinery to transmit intracellular signals, the TGFβ receptor complex.

TGFβ is secreted and bound in an inactive form to the latent TGFβ binding proteins (LTBPs) in the extracellular matrix. The mature, bioactive TGFβ is produced upon proteolytic cleavage of the latent complex by different proteases. The bioactive TGFβ binds to two

related receptors, namely TGFβRI and TGFβRII, the latter also named activin receptor-like kinase 5 (ALK5). Both receptors are serine/threonine kinases. Upon TGFβ binding, TGFβRI and TGFβRII form hetero-tetrameric complexes of two identical TGFβRI/TGFβRII receptors heterodimers. This leads to specific phosphorylation of serine and threonine residues in the juxtamembrane region of TGFβRI by TGFβRII. Subsequently, a conformational change of TGFβRI leads to the activation of its dormant catalytic domain and phosphorylation of TGFβRI associated effector proteins such as the receptor-associated Smad proteins (R-Smads). The activated R-Smads subsequently interact with the common mediator Smad 4, translocate into the nucleus and control the transcriptional expression of various genes.

Besides the well characterized canonical TGFβ signaling pathways is TGFβ also able to transmit signals via non-canonical signal transduction. This complex signaling machinery relies on the ability of the TGFβ-receptor complex to interact with various non-Smad interaction partners like receptor tyrosine kinases (RTKs) and diverse adapter proteins. Both, canonical as well as non-canonical TGFβ signaling can operate independently from each other, but mostly cooperate during TGFβ induced signaling. This leads to the immense complexity and variability of TGFβ signaling and its ability to control various cellular processes.

1.3.3. Canonical TGFβ signaling

Canonical TGFβ signaling utilizes Smad proteins to submit signals from the TGFβ-receptor complex to the nucleus. Ligand binding and activation of the TGFβ-receptor complex leads to the phosphorylation of Smad2/3 (R-Smads) at specific serine residues. These activated R-Smads form a trimeric complex with the common mediator Smad4, to enter the nucleus and interact with various transcriptional co-activators or co-repressors to control cellular processes like differentiation, cell-cycle arrest and apoptosis (described below). Within this basic signal transduction pathway, an intrinsic negative feedback loop is engaged to control canonical TGFβ signaling. This negative feedback is exerted primarily by three types of proteins: (i) the inhibitory Smads (i-Smads), (ii) ubiquitin ligases of the Smurf family and (iii) phosphatases like the SH2 domain-containing inositol 5-phosphatase (SHIP). The inhibitory Smad7 and the ubiquitin ligases Smurf1/2 are targets of canonical TGFβ signaling

and are rapidly upregulated. Smad7 is recruited to the activated TGFβ-receptor complex and inhibits TGFβ signaling by competing with R-Smads and by recruiting phosphatases like SHIP, which dephosphorylate and inactivate the TGFβ-receptor complex. Furthermore, Smad7 binds and activates Smurf ubiquitin ligases, leading to ubiquitinylation of the TGFβRI, thus provoking its endocytosis and lysosomal degradation (Di Guglielmo et al., 2003).

1.3.3.1. Canonical TGFβ signaling mediated cell-cycle arrest

In epithelial cells, TGFβ relays its cytostatic function via transcriptional activation of cyclin-dependent kinase (CDK) inhibitors, such as $p21^{CIP1}$ or $p15^{INK4B}$. The cytostatic function of both CDK inhibitors relies on their ability to inhibit the cell-cycle progression from the G1 to the S phase. This is achieved by preventing the complex formation and activation of cyclinD-cdk4/6 and cyclinE/A-cdk2 complexes by $p21^{CIP1}$ and $p15^{INK4B}$, respectively (Pardali et al., 2000; Gomis et al., 2006). Simultaneously, TGFβ inhibits cell-cycle progression by repression of c-Myc transcription, which plays an important role in cell growth and cell division. Upon canonical TGFβ signaling, c-Myc is downregulated by the coordinated activity of Smad3/4, retinoblastoma-like 1 (p107) and the transcription factors E2F4/5 and C/EBPβ (Chen et al., 2002; Gomis et al., 2006).

1.3.3.2. Canonical TGFβ signaling mediated apoptosis

Among the genes, which are regulated by TGFβ and are responsible for the induction of apoptosis, are GADD45β, the Bcl-2 homolog domain-only factor Bim, the death-associated protein kinase (DAPK) and SHIP. All these factors, except SHIP, promote apoptosis by inducing the release of mitochondrial cytochrome C and subsequent activation of caspase-mediated apoptosis. For example, GADD45β activates via mitogen-activated kinase kinase 4 (MKK4) the MAPK p38 and the pro-apoptotic protein Bad, which both cooperate to trigger mitochondrial cytochrome C release and the activation of caspase-mediated apoptosis. The lipid phosphatase SHIP promotes apoptosis by inhibiting PI3K activity, thereby blocking its survival promoting signaling (Zhang, 2009).

1.3.4. Non-canonical TGFβ signaling

The TGFβ-receptor complex interacts with various non-Smad signaling proteins, including receptor tyrosine kinases (RTKs), cytoplasmic adapter proteins and G-protein-coupled receptors. Each of these interaction partners can be activated upon TGFβ binding to the TGFβ-receptor complex and emit signals which can act independently or can interfere with canonical TGFβ signaling. Among well known signaling pathways which are triggered by non-canonical TGFβ signaling are the MAPK, PI3K and Rho-like GTPase signaling pathways (Zhang et al., 2009).

1.3.4.1. TGFβ-induced MAPK activation

Binding of TGFβ to the TGFβ-receptor complex leads to the trans- and autophosphorylation of TGFβRI and TGFβRII on multiple sites. These phosphorylation sites can be recognized by adapter proteins such as growth factor receptor binding protein 2 (Grb2) and Src homology domain 2 containing protein (Shc). Subsequent activation of Grb2 and/or Shc leads to activation of the small G-protein Ras, which in turn leads to the activation of the MAPK signaling pathway. Activation of the MAPK Erk1/2 during TGFβ-induced EMT was shown to be important for the disassembly of cell adherens junctions, modulation cell-matrix interactions, increased motility and endocytosis (Zavadil et al., 2001).

1.3.4.2. TGFβ-induced JNK/p38 activation

Another interaction partner of the activated TGFβ-receptor complex is the adapter protein and E3 ligase TRAF6. Binding of TRAF6 to the activated TGFβ-receptor complex induces polyubiquitination of TRAF6, and promotes its interaction with the TGFβ-activated kinase 1 (TAK1), followed by TAK1 polyubiquitination and its activation (Sorrentino et al., 2008; Yamashita et al., 2008a). TAK1 is than able to activate the MAPKKs MKK4 and MKK3/6, which in turn induce c-Jun N-terminal kinase (JNK) and MAPK p38 activation. This TRAF6-TAK1-JNK/p38 pathway is known to cooperate with canonical TGFβ signaling to induce apoptosis or EMT (Weston et al., 2007).

1.3.4.3. Rho-like GTPases in TGFβ-mediated EMT

Par6, a scaffold protein regulating epithelial cell polarity, interacts with TGFβRI at tight junctions. TGFβ stimulation induces the assembly and accumulation of the TGFβ receptor complexes at tight junctions, where TGFβRII phosphorylates Par6. Upon phosphorylation, Par6 recruits the E3 ubiquitin ligase Smurf1 to the activated receptor complex and mediates ubiquitination and turnover of RhoA, which finally enables TGFβ-dependent dissolution of tight junctions, a prerequisite for EMT (Ozdamar et al., 2005).

1.3.4.4. TGFβ-induced PI3K activation

TGFβRII was found to be continuously associated with p85, the regulatory subunit of PI3K. Upon TGFβ binding of the TGFβ-receptor complex, PI3K becomes activated leading to the phosphorylation of the PI3K target protein PKB and the activation of mammalian target of rapamycin (mTOR). mTOR is a key regulator of protein synthesis via phosphorylation of S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein (4E-BP1). The activation of S6K and 4E-BP1 by mTOR enhances translational capacity and protein synthesis, which is important for TGFβ-induced EMT. Furthermore, activation of PI3K protects from TGFβ induced death by (i) interfering with the apoptotic, canonical TGFβ signaling via phosphorylation and inhibition of the R-Smad Smad3 or (ii) inhibition of the transcription factor FoxO, which is essentially involved in TGFβ mediated cell-cycle arrest (described below) (Shin et al., 2001).

1.3.5. TGFβ as a pro-invasive factor

1.3.5.1. Myofibroblast Generation

The mobilization of myofibroblasts plays an important role in the pro-invasive function of TGFβ. TGFβ stimulates the generation of highly motile myofibroblasts from mesenchymal precursor cells, which possess features of fibroblasts and smooth muscle cells. These myofibroblasts, also described as cancer-associated fibroblasts, promote cancer cell invasion, aniogenesis and proliferation by producing pro-invasive and pro-angiogenic factors such as matrix metalloproteases, cytokines like interleukin 8 (IL-8), vascular endothelial growth factor (VEGF), and chemokines like CXCL12 (De Wever and Mareel, 2002; Allinen et al.,

2004).

1.3.5.2. Production of Autocrine Mitogens

TGFβ is able to promote tumor growth and invasion by stimulating the production of mitogenic factors. Gliomas which were able to attenuate the tumor-suppressive function of TGFβ benefit from an TGFβ-induced autocrine PDGF-B positive feedback loop. This is achieved by Smad-dependent transcriptional activation of the PDGF-B gene (Jennings and Pietenpol, 1998; Bruna et al., 2007).

1.3.5.3. Evasion of Immunity

TGFβ was shown to inhibit the anti-tumoral activity of cytotoxic $CD8^+$ T cells, by inhibiting their ability to produce cytolytic factors such as pore-forming protein perforin, the caspase-activating secreted factors granzyme A and B, and the pro-apoptotic cytokines Fas-ligand. In human glioma patients, TGFβ decreases the expression of the activating immunoreceptor NKG2D on $CD8^+$ T cells and natural killer (NK) cells, which leads to reduced $CD8^+$ T cells mediated cancer-directed cytotoxic response. Knockdown of TGFβ synthesis in a glioma cell lines prevented NKG2D repression and enhanced glioma killing by cytotoxic T cells and NK cells (Thomas and Massague, 2005).

1.3.5.4. Epithelial-Mesenchymal Transition

TGFβ belongs to most potent inducers of the epithelial-mesenchymal transition (EMT). Epithelial cells which undergo EMT loose E-cadherin-mediated intercellular connections, detach from the cell collective and change into individual, motile, protease-expressing mesenchymal cells. Cancer cells cells which undergo EMT are able to detach from the primary tumor, invade into the surrounding tissue and form metastatic lesions at distant sites. Interestingly, besides promoting invasiveness, TGFβ-induced EMT was shown to induce the transition of transformed and immortalized human mammary epithelial cells into mesenchymal cancer cells with stem cell traits, thus linking EMT to tumor cell plasticity (Shipitsin et al., 2007; Mani et al., 2008) .

1.4. EMT, the cytoskeleton, and cancer cell invasion (Review)

Mahmut Yilmaz and Gerhard Christofori: "EMT, the cytoskeleton, and cancer cell invasion". Cancer Metastasis Review 2009, 24.

Purpose of the review

The EMT-mediated gain of invasiveness requires a complex modulation of diverse biological processes such as intercellular adhesion, cell-matrix adhesion, extracellular matrix (ECM) production and cytoskeletal remodeling. In this review, we highlight new insights into various molecular and cellular processes occurring during EMT with the focus on the formation of invasive structures which are utilized by invading tumor cells to migrate and metastasize.

Summary

There is an increasing functional correlation between the formation of migratory and invasive membrane protrusions and the process of EMT. The structural as well as signaling molecules which are required for the formation of lamellipodia, filopodia and invadopodia are also found to be essentially required and to be active during EMT. Still, a direct link between the cellular and molecular processes underlying the formation of lamellipodia, filopodia and invadopodia on one hand, and EMT and tumor metastasis on the other hand, still remains to be established. If such link can be ascertained, the molecular mechanisms and genes and factors underlying the cellular organization of the cytoskeleton and the formation of membrane protrusions move into the spotlight of medical relevance, i.e. the design and development of innovative therapeutic approaches to interfere with cancer cell invasion and metastatic dissemination.

1.4.1. Epithelial-mesenchymal transition (EMT) and metastasis

Metastasis, the spread of tumor cells from a primary tumor to a secondary site within the human body remains one of the most life-threatening pathological events. In the last years, major efforts have been taken to understand the molecular mechanism underlying the distinct

steps of metastasis, which are (i) detachment of tumor cells from the primary tumor, (ii) invasion into surrounding tissue, (iii) intravasation into blood or lymphatic vessels, (iv) dissemination in the blood stream or the lymphatic system and, finally, (v) extravasation and outgrowth at a secondary site. Each of these steps requires a distinct molecular program in which the modulation of the adhesive and migratory and, thus, the cytoskeletal properties of the disseminating tumor cells play essential roles. To detach from the primary tumor and to invade into the surrounding tissue, tumor cells have to break down cell-cell contacts, remodel cell-matrix adhesion sites, and follow a chemoattractive path through the extracellular matrix, mined by secreted proteinases. These processes are commonly observed in various non-pathological conditions, such as in developmental processes like gastrulation or neural crest cell migration, where differentiated, epithelial cells dedifferentiate, move to a distant site, and then re-differentiate to form a new structure. This temporary and reversible phenomenon is known as the epithelial-mesenchymal transition (EMT), a process that is currently in the limelight of investigating the onset of cancer cell migration, invasion and metastatic dissemination (Grunert et al., 2003; Thiery and Sleeman, 2006). During EMT, non-motile, polarized epithelial cells, embedded via cell-cell junctions in a cell collective, dissolve their cell-cell junctions and convert into individual, non-polarized, motile and invasive mesenchymal cells. Thereby, the molecular repertoire of a cell experiences dramatic changes. For example, the function and expression of the epithelial cell-cell adhesion molecule E-cadherin is lost, whereas the expression of the mesenchymal cell-cell adhesion molecule N-cadherin is induced, a process also known as the cadherin switch. EMT can be prompted by various intrinsic signals (e.g. gene mutations) as well extrinsic signals (e.g. growth factor signaling). Among the growth factors known to induce EMT are transforming growth factor β (TGFβ)(Zavadil and Bottinger, 2005), hepatocyte growth factor (HGF)(Savagner et al., 1997), members of the epidermal growth factor (EGF) family (Lo et al., 2007), insulin-like growth factor (IGF)(Graham et al., 2008), and fibroblast growth factor (FGF)(Lee et al., 2006; Acevedo et al., 2007). Recently, also Notch signaling has been implicated in EMT in human breast cancer cells by activating the transcription factor Snail2 (Slug), a potent repressor of E-cadherin gene expression (Leong et al., 2007). Changes in the composition of

the extracellular matrix (ECM) are also able to induce EMT, as shown for collagen I and hyaluronan (Zoltan-Jones et al., 2003; Shintani et al., 2008b). With the diversity of signals inducing EMT the complexity of the interactive downstream effector pathways increases. Among the candidates which are engaged by TGFβ-induced EMT are the small GTPases RhoA and Rac1 (Bhowmick et al., 2001a; Bakin et al., 2002), Ras (Janda et al., 2002), phosphoinositol-3 kinase (PI3K)(Bakin et al., 2000), Mitogen-activated protein kinase (MAPK)(Bakin et al., 2002), integrin-linked kinase (ILK)(Lee et al., 2004), and the Jagged1/ Notch signaling pathway(Zavadil et al., 2004). With increasing interest in microRNAs, miR-200 and miR-205 have been recently shown to play an important role in TGFβ-induced EMT by modulating the function of ZEB1 (δEF1) and ZEB2 (Sip1), transcriptional repressors of E-cadherin gene expression (Gregory et al., 2008). Such complexity of interactive signaling upstream and downstream of the induction of EMT also explains why EMT is not a simple matter of changes in a cell's adhesive capabilities or its cytoskeletal organization, it rather represents a fundamental reprogramming of almost every aspect of a cell's biology. Still, the different signaling cascades underlying EMT can be grouped into biological programs and, apparently, tumor cells undergoing EMT hijack programs that are central for developmental processes. The actual occurrence of EMT in patients is still highly debated, yet with more detailed molecular and histopathological analysis and the advent of novel markers there is increasing evidence identifying EMT in various human cancers (Brabletz et al., 2005a; Sarrio et al., 2008). Still, many aggressive, invading tumors do not exhibit a molecular signature of EMT, suggesting that EMT may not be involved in every type of single cell invasion and that some tumors may undergo a partial or incomplete EMT (Tarin et al., 2005). In fact, cancer cells can invade in the absence of EMT and have a broad repertoire for invasion, including amoeboid or collective cell invasion (Friedl, 2004; Wicki et al., 2006). This review highlights recent novel insights into EMT research with a specific focus on the remodeling of the actin cytoskeleton and the formation of invasive structures during EMT and tumor cell invasion.

1.4.2. Epithelial cell-cell adhesion

The formation of a stable, polarized epithelium requires tight cell-cell and cell-matrix connections. E-cadherin is the major component of epithelial adherens junctions (AJ) which mediate, along with tight junctions, intercellular adhesion. AJ are located basal to the apical tight junctions (TJ) and form a belt-like structure which tie neighboring cells together (zonula adherens). E-cadherin is the prototype family member of classical cadherins, single-span transmembrane glycoproteins that interact in a calcium-dependent, homophilic manner with E-cadherins on neighboring cells. E-cadherin-mediated cell-cell adhesion complexes are anchored to the actin cytoskeleton via its cytoplasmic domain and β-catenin and α-catenin (Figure 3A). Thus, the formation of E-cadherin-mediated cell-cell adhesion fundamentally modulates the organization of cytoskeleton. This classical view of a direct connection between the E-cadherin/β-catenin/α-catenin complex and the actin network has been challenged by recent studies demonstrating that a reconstructed cadherin-catenin complex fails to bind actin filaments *in vitro* (Yamada et al., 2005), and that E-cadherin, localized in electron-dense microdomains called spot adherens junctions (SAJs), binds to actin in an α-catenin-independent manner (Cavey et al., 2008). Here, the authors describe a model in which two distinct actin populations are involved in the zonula adherens architecture (Figure 3A). One population represents stable, non-dynamic patches of highly organized actin to which the E-cadherin/β-catenin complex is attached in an α-catenin-independent manner (SAJs). The second population of actin is an underlying, dynamic actin framework to which the SAJs are linked and correctly positioned by α-catenin. One protein replacing α-catenin in the E-cadherin/β-catenin complex to SAJ could be eplin, a newly identified actin-binding protein(Abe and Takeichi, 2008). The juxtamembrane domain of E-cadherin binds to p120-catenin which is important in surface tracking, lysosomal degradation and correct membrane localization of E-cadherin (Thoreson et al., 2000; Ireton et al., 2002; Davis et al., 2003; Stehbens et al., 2006). Furthermore, p120-catenin plays an elementary role in the stability of epithelial cell-cell adhesion by repressing the activity of RhoA and activating Rac and Cdc42 (Noren et al., 2000; Noren et al., 2001; Wildenberg et al., 2006). All three GTPases are key regulators of actin assembly and play an essential role in the stability of cell-cell adhesion by

enforcing actin stress fibers (RhoA) and the formation of migratory membrane protrusions, such as lamellipodia and filopodia (Rac and Cdc42, respectively), as discussed below. Besides its adhesive function, E-cadherin also encompasses signaling capabilities, transduced predominantly by proteins interacting with its intracellular domain, such as β-catenin, or receptors that form multimeric complexes with E-cadherin, such as c-Met, the cognate receptor for HGF, IGF1R or integrins (Comoglio et al., 2003). Notably, E-cadherin has been shown to interact with a multimeric complex that consists of $\alpha_3\beta_1$-integrin, the tetraspanin CD151, which recruits protein kinase C-βII (PKCβII), receptor of activated protein kinase C-1 (RACK1), and the transmembrane protein tyrosine phosphatase PTPμ (Chattopadhyay et al., 2003) (Figure 3A). This multimeric complex promotes association of the cadherin-catenin complex with the actin cytoskeleton and supports cadherin mediated cell-cell adhesion. CD151 appears to be important for the filopodia-based "adhesion zipper formation", a process by which initial filopodia-mediated contacts of epithelial cells develop into mature cell-cell junctions (Vasioukhin et al., 2000; Shigeta et al., 2003). Moreover, CD151 expression accelerates E-cadherin-mediated intercellular adhesion by inducing Cdc42-induced filopodia extensions which form initial cell-cell contacts. Consistent with these observations, E-cadherin colocalizes and interacts with cortactin, a key regulator of actin-cytoskeleton assembly and remodeling (Helwani et al., 2004). E-cadherin also associates with c-Met, IGF1R and α_v-integrin at the plasma membrane (Reshetnikova et al., 2007; Canonici et al., 2008). Interestingly, in the absence of α-catenin the E-cadherin/IGF1R complex does not form, suggesting that α-catenin besides its function as actin-anchoring protein also exerts a function as an important scaffolding protein. Upon stimulation with IGF-I, α_v-integrin dissociates from the cell-cell adhesion complex and translocates to focal contact sites of invasive structures, such as invadopodia (see below). These findings expand E-cadherin's functional repertoire beyond its adhesive functions and emphasize the critical role of E-cadherin as a regulator of signaling complexes.

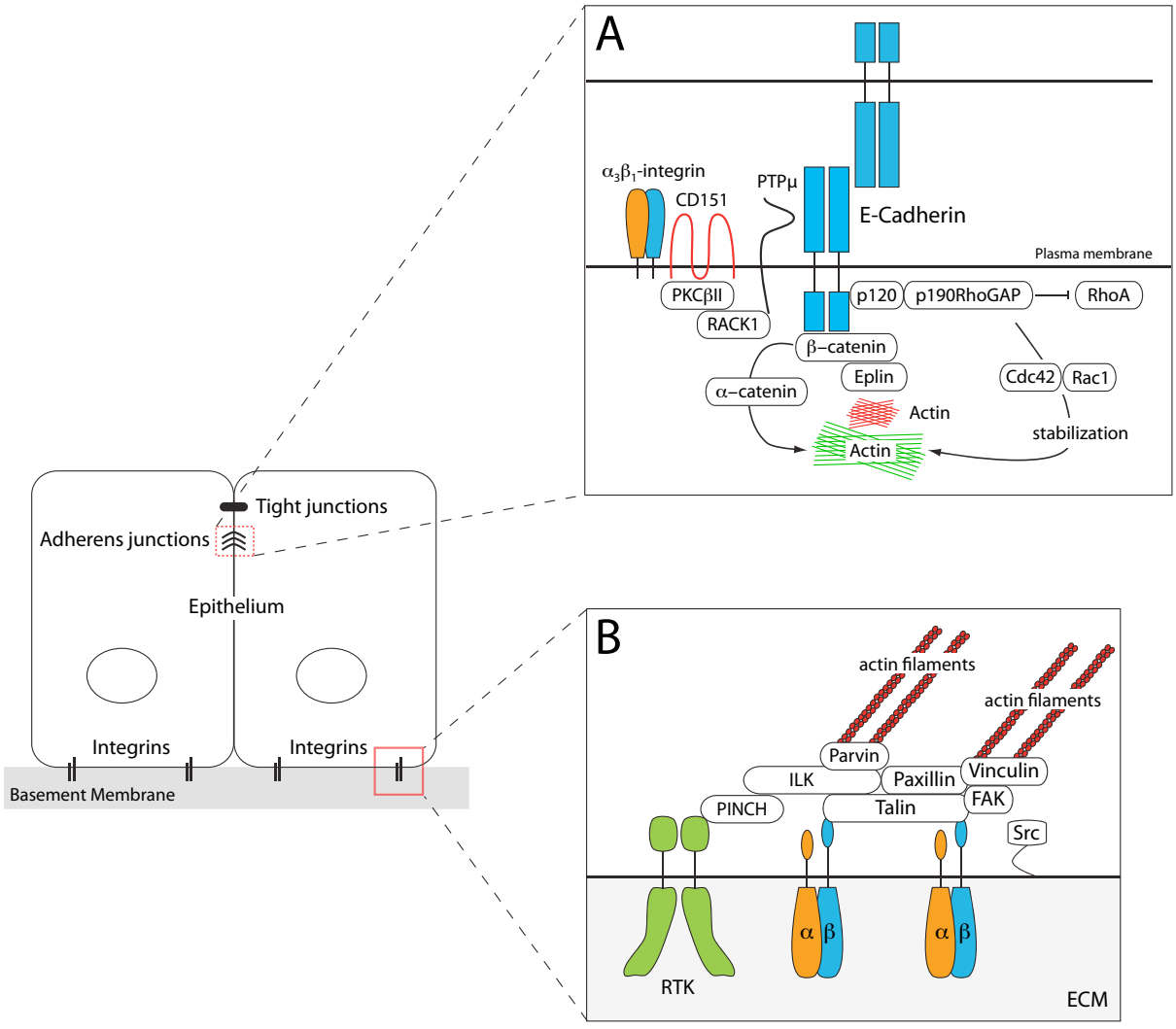


Figure 3. Differentiated, polarized epithelial cells are tightly attached to their neighboring cells by E-cadherin-mediated cell-cell adhesion complexes and to the extracellular matrix via integrins. **(A) Epithelial cell-cell adhesion.** E-cadherin is connected via β-catenin and Eplin to stable, electron-dense actin microdomains called spot adherens junctions (SAJ) in an α-catenin-independent manner. These E-Cadherin-SAJs complexes are attached and correctly positioned to an underlying dynamic actin framework via α-catenin. E-cadherin-mediated cell adhesion is stabilized by (i) p120-catenin recruited p190RhoGAP, which stabilizes the actin cytoskeleton underlying the adhesion complex by balancing the activities of RhoA and Rac1/Cdc42, and by (ii) protein tyrosine phosphatase PTPμ, which keeps β-catenin in an dephosphorylated state thereby preventing its degradation. PTPμ is correctly positioned to β-catenin by a multimeric protein complex consisting of receptor of activated protein kinase C-1 (RACK1), protein kinase C-βII (PKCβII), the tetraspanin CD151 and $\alpha_3\beta_1$-integrin. **(B) Epithelial cell-matrix adhesion.** Integrin-mediated cell-matrix adhesion and linkage to the actin cytoskeleton is accomplished by a multiprotein complex consisting of the adaptor proteins talin, paxillin, vinculin and the ternary complex of pinch, parvin and integrin-linked kinase (ILK), called tIPP complex. The interaction and phosphorylation status of focal adhesion kinase (FAK) and the non-receptor tyrosine kinase Src is critical for integrin complex assembly and turnover.

1.4.3. Loss of E-cadherin function and its consequences

Loss of E-cadherin gene expression or of E-cadherin protein is frequently found during tumor progression in most epithelial cancers. Hence, loss of E-cadherin function is a clinical indicator for poor prognosis and metastasis (Perl et al., 1998; Bissell and Radisky, 2001; Cavallaro and Christofori, 2004). Since E-cadherin plays a key role in epithelial structure and homeostasis its expression underlies a strict control. As many other proteins, E-cadherin can be regulated at the transcriptional as well as at the post-translational level, yet both mechanisms usually cooperate for an efficient repression of E-cadherin function.

1.4.3.1. Transcriptional control of E-cadherin

Transcriptional repression of E-cadherin is mediated by a list of transcription factors, among them intensely studied factors like Snail1 (Snail), Snail2 (Slug), ZEB1 (δEF1), ZEB2 (Sip1), E47, and Twist (Peinado et al., 2007) (Figure 4A). The expression of these repressors can be induced by a variety of stimuli, including activation of the TGFβ, HGF, EGF, Wnt, and Notch signaling pathways. Moreover, they seem to regulate each other's expression in positive and negative feedback loops. Engagement of these transcriptional repressors at the E-cadherin gene promoter eventually leads to epigenetic silencing of the gene by histone modifications (acetylation, methylation, phosphorylation, ubiquitinylation, sumoylation) and subsequently by DNA hypermethylation (Jenuwein and Allis, 2001; Zhang and Reinberg, 2001; Kouzarides, 2007). Such silencing of the E-cadherin promotor is a complex process (Herranz et al., 2008). As a first step, Snail1 recruits the histone deacetylase HDAC to the E-cadherin promotor complex, thereby inducing histone deacetylation. Subsequently, the polycomb repressor complex 2 (PRC2) is recruited to the site and methylates histones, thus supporting E-cadherin repression. Upon initial down-regulation of E-cadherin gene expression, Snail1 induces ZEB1 expression, which in turn engages a second, PRC2-independent repressor complex that further inhibits E-cadherin expression. In addition, new interaction partners of Snail1 have been identified, such as the LIM-domain protein Ajuba which recruits the protein arginine methyltransferase 5 (PRMT5) to support Snail1-mediated transcriptional repression (Hou et al., 2008). In a large variety of human cancers, the E-

cadherin gene is found to be highly hypermethylated, yet how the initial silencing of the gene promoter converts into a more long-term repression by DNA hypermethylation remains to be resolved (Berger, 2007). In the context of EMT and the metastatic dissemination of tumor cells, the molecular basis of the reversibility or irreversibility of E-cadherin's epigenetic silencing is thus a future challenge. This complexity and/or variability in E-cadherin regulation indicates that the cell has a dynamic range of E-cadherin expression and depending on the actual need can either totally suppress or temporally attenuate its expression.

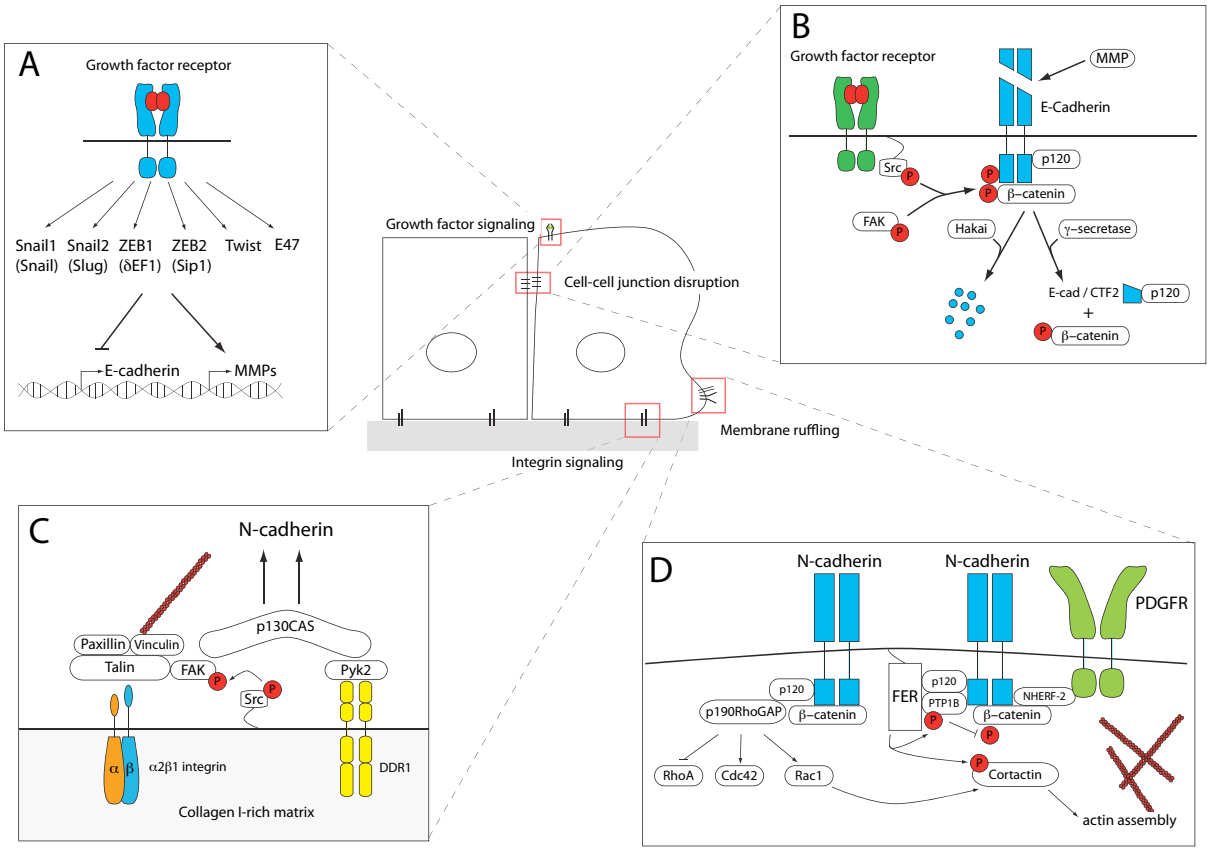


Figure 4. Induction of epithelial-mesenchymal transition (EMT) (A) Repression of E-cadherin gene expression. Growth factor stimulation induces various signaling cascades leading to the induction of EMT. Among the regulated genes are transcription factors that repress E-cadherin gene expression and induce expression of genes such as matrix metalloproteinases (MMP). **(B) Loss of E-cadherin function.** The E-cadherin cell-cell adhesion complex is destabilized by Src and/or FAK- mediated phosphorylation of either E-cadherin or β-catenin. The phosphorylation of the cytoplasmic E-cadherin domain induces its proteasomal degradation mediated by the E3-ubiquitin-ligase Hakai. Phosphorylation of β-catenin induces its detachment from E-cadherin and relocalization into the nucleus, where together with Tcf transcriptions factors modulates the expression of pro-invasive genes. Intracellular cleavage of E-cadherin by γ-secretase also results in a loss of E-cadherin function, yet its newly generated C-terminal fragment (CTF2) together with p120-catenin translocates into the nucleus, where it modifies the activity of the transcriptional repressor Kaiso. **(C) Integrin-mediated support of EMT.** Enrichment of collagen I in the extracellular matrix supports EMT via the coordinated signaling of β_1-integrins and the collagen receptor DDR1. These signals converge via FAK and Pyk2 kinases on the $p130^{Crk}$-associated scaffold (CAS) protein, resulting in the induction of N-cadherin expression. **(D) The formation of protrusive membrane structures.** Upon PDGF stimulation, N-cadherin co-localizes with PDGFR in membrane ruffles. The interaction of both N-cadherin and PDGFR relies on Na^+/H^+ exchanger regulatory factor-2 (NHERF-2) which is recruited to the complex by p120-catenin. Localized in membrane ruffles, N-cadherin contributes to actin cytoskeleton remodeling (i) by recruiting p190RhoGAP and thereby favoring Rac1 and or Cdc42-mediated actin remodeling and (ii) by employing p120-catenin to recruit the non-receptor tyrosine kinase Fer. Fer in turn phosphorylates and activates cortactin which induces actin assembly. Morover, Fer stabilizes the N-cadherin complex by phosphorylating and activating protein tyrosine phosphatase PTP1B, thus preventing β-catenin phosphorylation and degradation.

1.4.3.2. Post-translational control of E-cadherin

On the post-translational level, the transport of newly synthesized E-cadherin to the cell membrane can be inhibited via O-glycolysation (Zhu et al., 2001) or mature, membrane-bound E-cadherin can be degraded by proteolytic cleavage or endocytosed from the plasma membrane (Lochter et al., 1997; Steinhusen et al., 2001; Marambaud et al., 2002; Maretzky et al., 2005). The proteolytic cleavage of E-cadherin can also produce E-cadherin fragments which exert signaling functions. For example, γ-secretase-mediated cleavage of E-cadherin produces a C-terminal, cytoplasmic fragment (CTF2) that is transported into the nucleus in a p120-catenin-dependent manner (Figure 4B). In the nucleus, CTF2 modulates the interaction between p120-catenin and Kaiso, a transcriptional repressor, thereby affecting for example cell survival (Ferber et al., 2008). Destabilization of the E-cadherin adhesion complex is also accomplished by receptor tyrosine kinase or Src-mediated phosphorylation of E-cadherin, followed by its ubiquitylation by the E3 ligase Hakai and subsequent degradation (Gumbiner, 2000; Fujita et al., 2002) (Figure 4B). Moreover, integrin-activated focal adhesion kinase (FAK) can phosphorylate β-catenin and thus induce its ubiquitylation and degradation and the disassembly of the E-cadherin cell adhesion complex (Koenig et al., 2006). Endocytosis of E-cadherin can occur via clathrin or caveolin-dependent mechanisms (Akhtar and Hotchin, 2001; Lu et al., 2003; Janda et al., 2006). A key player in clathrin-mediated E-cadherin endocytosis is Arf6, a Ras-related small GTPase. It promotes endocytosis via recruitment of Nm23-H1, a nucleoside diphosphate kinase (and the first metastasis suppressor gene ever identified (Steeg et al., 1988)), which in turn activates dynamin-dependent fission of vesicles and destabilization of cortical actin by recruiting the guanine nucleotide exchange factor (GEF) Tiam1, a Rac1 inhibitor (Palacios et al., 2002). Recently, a new GTPase activating protein (GAP) for Arf6, Smap1, has been identified, which plays an essential role in E-cadherin endocytosis, revealing a new player in post-translational E-cadherin control (Tanabe et al., 2005; Kon et al., 2008). The downregulation of E-cadherin not only leads to a mechanical disruption of AJ, it also liberates proteins from the cytoplasmic cell adhesion complex which exert ambivalent functions depending on their subcellular localization. The probably most prominent cytoplasmic interaction partner of E-cadherin is β-catenin, well

known for its dual role in cell adhesion and Wnt signaling (Clevers, 2006). Stabilized by active Wnt signaling or by mutations in the β-catenin phosphorylation/degradation pathway, β-catenin accumulates in the cytoplasm and enters the nucleus where it interacts with members of the Tcf/Lef family of transcription factors and modulates expression of a large number of genes involved in cell proliferation, migration, invasion, and morphogenesis, including cyclin D1, the cell adhesion molecule L1-CAM, matrix metalloproteases (MMP) and the metastasis gene S100A4 (Arce et al., 2006) (Wong and Pignatelli, 2002). Another recently discovered target of β-catenin/Tcf signaling is Fascin, a actin-bundling protein which is essential for filopodia formation and cancer cell invasion (Vignjevic et al., 2006; Vignjevic et al., 2007). Similar to β-catenin, upon loss of E-cadherin function, p120-catenin is also freed from the cytoplasmic cell adhesion complex and accumulates in the cytoplasm. In addition to its functions on Rho family GTPases and the actin cytoskeleton (see above), p120-catenin can traffic to the nucleus where it binds to the transcriptional repressor Kaiso. In contrast to β-catenin/Tcf-mediated transcription, where β-catenin acts as a transactivator, p120-catenin has no transactivation domain and rather releases Kaiso from its promoter binding sites and thus activates gene expression by de-repression. However, the nature of p120/Kaiso target genes is still poorly defined (van Roy and McCrea, 2005).

1.4.4. The cadherin switch and its consequences

The loss of epithelial E-cadherin and the gain of mesenchymal N-cadherin expression is a major hallmark of EMT. This cadherin switch leads to a drastic change in the adhesive properties of a cell, as it loses its affinity for epithelial neighbors and gains affinity for mesenchymal cells, such as fibroblasts or vascular endothelial cells. Besides the change of the adhesive repertoire, the gain of N-cadherin expression also provokes increased cell migration and invasion (Nieman et al., 1999; Hulit et al., 2007). Like E-cadherin, N-cadherin belongs to the family of classical cadherins and forms homophilic cell-cell adhesion junctions. N-cadherin is normally expressed in nervous tissue, in vascular endothelial cells, and in skeletal and cardiac muscle cells. Its expression is upregulated in the cells of the primitive streak during mesoderm formation and during progression of a variety of cancers,

where its expression correlates with poor prognosis (Hazan et al., 2004; Gravdal et al., 2007; Hulit et al., 2007). Investigations into the molecular mechanisms underlying the induction of N-cadherin expression during EMT have only recently begun. It has been shown that N-cadherin can be upregulated by collagen I via the coordinated engagement of the collagen receptor discoidin domain receptor 1 (DDR1) and $\alpha_2\beta_1$-integrin based on a $p130^{Crk}$-associated substrate (CAS) scaffold (Shintani et al., 2008a; Shintani et al., 2008b) (Figure 4C). On the transcriptional level, the transcriptional repressor Twist appears to be involved in the induction of N-cadherin gene expression in an β_1-integrin-dependent manner (Alexander et al., 2006; Niu et al., 2007; Yang et al., 2007). Like its epithelial counterpart, N-cadherin is connected via α-catenin and β-catenin to the cytoskeleton and functions as both a mechanical cell adhesion component and a signaling molecule. Recently, it has been reported that during neurite extension the traction forces generated by retrograde actin flow are directly transmitted to N-cadherin adhesions, thereby mechanically linking N-cadherin with the formation of motile structures (Bard et al., 2008). Interestingly, the authors show that the mechanical engagement of N-cadherin induces local actin polymerization and thereby ensures the integrity of the adhesion complex. Engagement of N-cadherin activates the RhoGTPase Rac1, which in turn recruits the actin remodeling protein cortactin to the N-cadherin adhesion complex (El Sayegh et al., 2005) (Figure 4D). Within this complex, the non-receptor tyrosine kinase Fer associates with N-cadherin via p120-catenin (Kim and Wong, 1995). Fer then phosphorylates and activates cortactin, thereby inducing actin remodeling, and increasing the mobility of N-cadherin molecules to extend the adhesion zone and, finally, to promote the formation of stable cell-cell adhesion. In line with these observations, N-cadherin is found to be localized in the lamellipodia of adjacent, contacting myoblasts (Comunale et al., 2007). Fer also phosphorylates the phosphatase protein tyrosine phosphatase 1B (PTP1B) and promotes its binding to N-cadherin. The PTP1B/N-cadherin interaction protects β-catenin from degradation by keeping it in a dephosphorylated state and thereby ensures the stability of the N-cadherin-mediated cell-cell junctions (Xu et al., 2002; Xu et al., 2004) (Figure 4D). Also similar to E-cadherin, N-cadherin interacts with a number of signal transduction molecules and contributes to various signaling pathways. For example,

Na+/H+ exchanger regulatory factor (NHERF)-2 has been shown to physically link N-cadherin to PDGF receptor (PDGFR) by binding both β-catenin in the N-cadherin/β-catenin complex and PDGFR (Theisen et al., 2007) (Figure 4D). PDGFR activation is known to induce actin reorganization and cell proliferation and differentiation and to play an important role in EMT (Heldin et al., 1998; Kong et al., 2008). Interestingly, PDGF stimulation of NIH3T3 cells leads to colocalization of N-cadherin, p120-catenin and p190RhoGAP in dorsal circular ruffles (DCRs), structures known to depend on growth factor-induced Rac activity and subsequent RhoA inhibition (Ridley et al., 1992; Sander et al., 1999; Pertz et al., 2006). This is interesting because p120-catenin is not only important for the recruitment of Fer to the N-cadherin complex, but together with p190RhoGAP also coordinates the antagonistic functions between Rac and RhoA (Nimnual et al., 2003; Wildenberg et al., 2006). This antagonism plays a critical role in defining the structure of the actin cytoskeleton. The active form of RhoA stimulates focal-adhesion (FA) formation and contractility via assembly of predominantly radially-oriented actin stress fibers (ASF), whereas Rac activation induces cell spreading, migration and membrane ruffling via actin polymerization at the cell periphery. Moreover, Rac activation inhibits Rho activity, which can also be achieved by p120-catenin over-expression (Anastasiadis et al., 2000) (Figure 4D). Besides PDGR, N-cadherin also interacts with FGF receptors (FGFR) in a complex with neural cell adhesion molecule (NCAM), a immunoglobulin domain cell adhesion molecule (Cavallaro et al., 2001; Cavallaro and Christofori, 2004). The interaction between N-cadherin and FGFR leads to stabilization of FGFR at the membrane surface by preventing its internalization upon ligand binding. As a result, sustained MAPK pathway activation and increased cell motility and MMP secretion promote invasiveness of N-cadherin-expressing cells (Hazan et al., 2000; Williams et al., 2001; Suyama et al., 2002). NCAM accomplishes a similar induction of cell migration and invasion by directly binding and stimulating FGFR via its fibronectin type III domains (Cavallaro et al., 2001). Thereby, NCAM-mediated stimulation of FGFR signaling differs substantially from FGF-induced FGFR signal transduction resulting into different cellular outcomes, such as increased cell-substrate adhesion, migration and invasion by NCAM and cell proliferation by FGF (Francavilla et al., 2007). The interaction between N-

cadherin and NCAM with FGFR relies on a CAM-domain proximally located to the acid box region of the FGFR, which is not required for FGF ligand binding (Sanchez-Heras et al., 2006). Such complex interactions between different cell adhesion molecules and tyrosine kinases raise another level of complexity in the regulation of cell migration, invasion and metastasis formation. For a more detailed insight into specific aspects of cell adhesion and signaling complexes at the invasive cancer front we refer the reader to recently published reviews (Cavallaro and Christofori, 2004; Christofori, 2006). Like E-cadherin, N-cadherin is also proteolytically processed to generate shedded extracellular domain and intracellular domain fragments with potential signaling functions. In neurons, N-cadherin is cleaved by ADAM10 and by PS1/γ-secretase to produce a cytoplasmic fragment of N-cadherin, N-Cad/CTF2, for example stimulated by bone morphogenic protein-4 (BMP4) (Marambaud et al., 2003; Shoval et al., 2007). N-Cad/CTF2 is able to interfere with the CPB/CREB transcription complex, by binding the transcription factor CBP and inducing its proteasomal degradation. N-Cad/CTF2-induced repression of CPB/CREB-mediated transcriptional control suppresses expression of genes important for proliferation and differentiation, such as c-Fos. N-Cad/CTF2 also promotes migration of neural crest cells by increasing the expression of β-catenin and therewith inducing the expression of β-catenin target genes like cyclin D1. Interestingly, N-Cad/CTF2 facilitates β-catenin-dependent signaling (i) by inhibiting β-catenin phosphorylation, (ii) by increasing β-catenin transcription and (iii) by reducing full-length N-cadherin protein levels to prevent sequestration of β-catenin to cell-cell junctions (Uemura et al., 2006). These data describe N-cadherin as a critical protein in the regulation of EMT and cell invasiveness. Besides simply exercising a cell adhesion function that changes a cell's affinity from epithelial cells to mesenchymal cells, it is actively involved in delineating a cell's migratory state by modulating growth factor signaling and remodeling the actin cytoskeleton. Such pleiotropic functions certainly warrant detailed future experimental investigations.

1.4.5. Integrin-mediated cell-matrix adhesion and signaling

The extracellular matrix (ECM) is a constantly remodeled 3D structure consisting of a variety

of specialized proteins and proteoglycans which are able to regulate many cellular processes, including cell proliferation, survival, differentiation, and migration. This control is mainly based on a constant communication between the adhesive adaptors of a cell, integrins, and the ECM. Changes of a cell's integrin repertoire or the composition of the ECM can have drastic consequences for the cell and, in extremis, can lead to cell death as well as transformation. Besides its scaffolding function, the ECM is also able to bind and sequester diverse growth factors and chemokines which can be retrieved by local proteolysis and, dependent on activation or inactivation, affect cell behavior. Integrins are heterodimeric type-I transmembrane proteins consisting of an α-and a β-chain. Mammals have 18 α and 8 β chains which are combined to generate 24 different combinations which bind in a specific, yet partially overlapping, manner various components of the ECM. Similar to cadherins, also integrins function as both mechanical adhesion and signaling molecules. Importantly, integrins switch between an inactive, low ligand-affinity conformation and an active, high ligand-affinity conformation. Activation can be achieved by binding of intracellular proteins to integrins, such as talin, or by MMP-mediated proteolytic cleavage (Deryugina et al., 2000; Tadokoro et al., 2003). Integrins are linked to the actin cytoskeleton and signal via the ternary complex of integrin-linked kinase (ILK), pinch and parvin, also named the tIPP complex (Legate et al., 2006) (Figure 3B). In the tIPP, integrins are linked through ILK to the actin cytoskeleton, either via parvin itself or via a paxillin/vinculin/parvin complex. ILK plays a major role as a scaffold protein in assembling the multimeric protein complex which is necessary for the integrin-actin cytoskeleton linkage (via parvin, paxillin and vinculin). Moreover, ILK is required for the formation of signaling complexes with receptor tyrosine kinases via pinch. The signaling cascades triggered by the activation of integrins and its cytoplasmic partners are complex. Among the direct targets downstream of integrins are FAK, Src-family kinases, glycogen synthase-kinase-3β (GSK3β) and protein kinase B (AKT/PKB). Effectors of these signaling cascades include MAPK, NFκB, Jun, β-catenin and others and together they modulate cell proliferation, cell survival, cell migration and invasion. Among the mitogenic integrins are $\alpha_6\beta_4$ or $\alpha_v\beta_3$-integrins which cooperate with diverse growth factor receptors, including EGFR, ErbB2, and c-Met (Gambaletta et al., 2000;

Mariotti et al., 2001; Mercurio and Rabinovitz, 2001; Trusolino et al., 2001). On the other hand, growth inhibitory signals can be transmitted, for example via $\alpha_2\beta_1$-integrin, which in turn activates p38-MAPK-mediated cell-cycle inhibition (Ellinger-Ziegelbauer et al., 1999; Ivaska et al., 1999). Integrin $\alpha_v\beta_6$ or $\alpha_v\beta_8$ engagement leads to the activation of latent TGFβ thereby executing its cytostatic effect (Munger et al., 1999; Mu et al., 2002). Faced by this dichotomy of integrin signaling, cancer cells switch their integrin expression to a pro-oncogenic repertoire in order to invade and survive in the surrounding tissue.

1.4.5.1. Integrins in EMT and cell invasion

The function of integrins during EMT is diverse and dynamic as they are able to initiate and enforce EMT and invasion. For example, engagement of integrins $\alpha_1\beta_1$ or $\alpha_2\beta_1$ by collagen type I results in a loss of E-cadherin mediated cell-cell contacts, along with the activation of the β-catenin/Tcf pathway in pancreatic cancer cells (Koenig et al., 2006; Wipff and Hinz, 2008). Furthermore, as described above, collagen type I is also able to induce N-cadherin expression upon activation of the integrin $\alpha_2\beta_1$ together with the collagen receptor discoidin domain receptor 1 (DDR1), a receptor tyrosine kinase (Shintani et al., 2008a). Both, the downregulation of E-cadherin and upregulation of N-cadherin play important roles in the initiation and execution of EMT. Interestingly, Snail1, the transcriptional repressor of E-cadherin expression and a potent inducer of EMT, is able to induce the expression of $\alpha_v\beta_3$-integrin which is well known for its pro-invasive functions and its localization in the invading front of cancers (Sharma and Henderson, 2007; Haraguchi et al., 2008). Along with their function as mechanical anchor proteins for cell migration and invasion, integrins play an important role in the correct localization of proteases. Several reports demonstrate that the colocalization and cooperation of β1-integrin and MT1-MMP1 is necessary for cancer cell invasion into a collagen matrix and that both MT1-MMP and β_1-integrins have important roles in EMT (Pulyaeva et al., 1997; Bhowmick et al., 2001b; Ellerbroek et al., 2001; Wolf et al., 2003b; Cao et al., 2008). The localization of MT1-MMP to β_1-integrins is an exocytic event dependent on the activity of the GTPase Rab8 (Bravo-Cordero et al., 2007). Integrin-mediated recruitment of ECM remodeling proteases is also responsible for the liberation and/

or activation of matrix bound growth factors and chemokines. As described above, the cooperation of MT1-MMP and $\alpha_v\beta_8$-integrin leads to the activation of latent TGFβ (Mu et al., 2002; Sheppard, 2005). TGFβ usually acts as a cytostatic, tumor suppressing factor, but it promotes tumor progression and invasion, if the tumor cells overcome its cytostatic and apoptotic effects (Roberts and Wakefield, 2003). In fact TGFβ is one of the most potent inducers of EMT in cultured cells *in vitro* and in animal models *in vivo* (Thiery and Sleeman, 2006). Other integrins which are upregulated during EMT, such as $\alpha_v\beta_6$-integrin, are also able to increase protease expression and to liberate and activate TGFβ (Bates et al., 2005; Bates, 2005; Araya et al., 2006). Also, the activities of the cytoplasmic interaction partners of integrins, ILK and pinch, have been implicated in the process of EMT (Oloumi et al., 2004; Bagnato and Rosano, 2007; Li et al., 2007).

1.4.6. EMT and the actin cytoskeleton

The actin cytoskeleton is a highly dynamic structure, which is constantly remodeled in a living cell. This dynamics are based on a well-balanced and highly controlled equilibrium of local assembly and disassembly of actin filaments. Obviously, such regulation is a prerequisite for processes like endocytosis, cell motility, and cancer cell invasion.

1.4.7. RhoGTPases and EMT

The members of the Rho GTPase family are mainly responsible for integrating and transmitting signals from chemokine and growth factor receptors and from adhesion receptors to effector proteins of actin remodeling. RhoGTPases are activated upon GTP binding and inactive in their GDP-bound form. RhoGTPase activation is tightly controlled by three groups of regulatory proteins, guanine nucleotide exchange factors (GEF), GTPase-activating proteins (GAP), and guanine nucelotide dissociation inhibitors (GDI). GEF are responsible for the activation of RhoGTPases by promoting the exchange of Rho-bound GDP by GTP. This is counteracted by GAP which raise the intrinsic GTPase activity of RhoGTPases and the hydrolysis of bound GTP to GDP. Finally, GDI bind inactive Rho-GDP and prevent the interaction with RhoGEFs and thus its activation. RhoA, Rac1 and Cdc42 are best studied

among the 23 family members of RhoGTPases. The complexity of RhoGTPase signaling arises not only from the size of its family members and number of effector proteins (~ 70 proteins), but also from the numbers of GEF (~ 70 members), GDI (~3 members) and GAP (~ 60 members) which modulate their activity. Depending on which GEF, GDI or GAP is interacting with the RhoGTPase the biological response can be different. In the GTP-bound form, RhoGTPases activate effector proteins, which are often serine/threonine kinases, such as the p21-activated kinases (PAK) for Rac1 and Cdc42 and the ROCK kinases for RhoA. In general, RhoGTPases affect almost all cell biological processes in a cell's life (Etienne-Manneville and Hall, 2002; Sahai and Marshall, 2002; Burridge and Wennerberg, 2004). With regard to migration and invasion and in a simplified view, RhoA induces actin stress fiber formation and regulates cytoskeletal changes affecting cell-cell or cell-matrix adhesion, Rac1 is involved in lamellipodia and membrane ruffle formation, and Cdc42 is involved in filopodia formation (Hall, 2005; Ridley, 2006)**.** Based on their central function in actin remodeling and their ability to induce MMPs, Rho GTPases play an important role in EMT as well (Lozano et al., 2003),. During growth factor-induced EMT, tight control of the activities of RhoGTPases is critical. As mentioned above, depending on the presence of epithelial or mesenchymal cadherins, the localization and function of p120-catenin and thus the activity of RhoGTPases change dramatically (Cozzolino et al., 2003). In epithelial cells, p120-catenin localizes at the cell membrane and associates with E-cadherin where it controls the activity of RhoA and Rac1. RhoA activity, which is required for the initial cell-cell contact formation, is downregulated in established, mature cell adhesions. Both, activation and inactivation of RhoA require the p120-catenin-dependent recruitment of RhoGEFs, like Vav2, or RhoGAPs, like p190-RhoGAP, respectively. The recruitment of p190-RhoGAP results in the activation of Rac1 which leads to a stabilization of E-cadherin junctions by inhibiting the activities of IQ-domain GTPase-activating protein 1 (IQGAP1), a Rac1 effector protein and a mediator of E-cadherin endocytosis (see also below). Moreover, the actin cytoskeleton underlying cell contacts is reorganized and stabilized (Anastasiadis, 2007). During EMT, p120-catenin binds to mesenchymal cadherins at the cell membrane but is also found localized in the cytoplasm. Cytoplasmic p120-catenin functions as a RhoA-GDI that

binds and represses RhoA activity (Bellovin et al., 2005). Simultaneously, p120-catenin bound to mesenchymal cadherins at the cell membrane promotes Rac1 activity and induces the formation of motile, protrusive membrane structures, such as lamellipodia. Thus, both cytoplasmic and membrane-sequestered p120-catenin cooperate to induce cell motility during EMT. Interestingly, Rac1 inhibits RhoA activity by inducing the production of reactive oxygen species (ROS), which in turn activate p190RhoGAP by inhibiting the low-molecular weight protein tyrosine phosphatase (LMW-PTP)(Sander et al., 1999; Zondag et al., 2000). Moreover, the expression of Snail1, the transcriptional repressor of E-cadherin gene expression and potent inducer of EMT, is is increased upon Rac1-mediated ROS production (Radisky et al., 2005). The importance of RhoGTPases in EMT is also underscored by the notions that Rac1b, a splice variant of Rac1, is highly expressed in malignant breast tissue, that RhoA downregulation is required for EMT in colon carcinoma progression, and that RhoC activity is critical for cell migration, invasion and metastasis after EMT (Clark et al., 2000; Bellovin et al., 2005; Hakem et al., 2005). The activity of RhoA during EMT not only effects cell-cell adhesion but also microtubule-mediated cell-matrix adhesion and basement membrane integrity (Nakaya et al., 2008). RhoA, when localized at the cell-basement membrane (BM) gets in contact with Net1, a RhoA-specific GEF, thereby exerting an important function for the integrity of the BM in epiblasts. During gastrulation, a process resembling EMT, RhoA at the cell basis loses its activity, which leads to microtubule destabilization, cell-BM contact disruption and BM breakdown. Notably, destabilization of the cell-BM contacts precedes breakdown of cell-cell adhesions. Tiam1, a GEF for Rac1, also exerts a critical function in both E-cadherin-mediated cell-cell junction stability and during EMT. Loss of Tiam1 activity is required for the induction of EMT; forced expression of constitutive-active forms of Rac1 (RacV12) or Tiam-1 prevents HGF-induced EMT in epithelial cells (Hordijk et al., 1997; Malliri et al., 2004). Interestingly, ablation of Tiam-1 in a mouse model of chemical-induced skin carcinogenesis reduces tumor incidence yet increases tumor malignancy, demonstrating the ambivalent role of Rac1 in tumor formation and tumor progression (Malliri et al., 2002). Despite major progress, the detailed role of Rho family GTPases in EMT and tumor progression still remains unresolved. The sophistication

of tumor cell motility and invasion on one side and the immense complexity of the regulation of RhoGTPases on the other side obscure a simple solution. The formation of membrane protrusions and other RhoGTPase-dependent activities during EMT are not linear processes, and rather depend on the integrated activities of many members of the RhoGTPase family and their interaction with various regulatory proteins which will eventually determine the time and localization of their specific activities.

1.4.8. Membrane ruffles

The onset of motility requires a relaxation of static actin structures in order to form pliable membrane protrusions. Rigid actin stress fibers are disassembled upon dorsal circular ruffle (DCR) formation leaving a fine cortical actin meshwork behind, from which cell membrane protrusions like lamellipodia can emerge (Ballestrem et al., 1998; Krueger et al., 2003). DCRs are short-lived actin structures formed at the dorsal surface of growth factor (PDGF, HGF, EGF)-stimulated cells. DCRs, formed at the leading edge of growth factor-stimulated cells, are also able to secrete MMP (e.g. MMP-2), revealing their potential role for the onset of cell invasion (Suetsugu et al., 2003). Besides relaxing static membrane structures, DCRs are also important for macropinocytosis in growth factor-stimulated epithelial cells (Vieira et al., 1996; Orth and McNiven, 2006). Macropinocytosis plays an important role in the modulation of cell signaling; it may either inhibit signal transduction via degradation of growth factor receptor complexes or it may potentiate signaling, as shown in the case of EGF (Vieira et al., 1996). DCRs are enriched with actin assembly proteins, such as Arp2/3, WASP and cortactin, suggesting that DCR formation requires actin assembly. As mediators of growth factor signaling, RhoGTPases, like Rac1 or Cdc42, are required for DCRs formation as well (Dharmawardhane et al., 2000). Other reports indicate the involvement of protein kinase A (PKA) and the cytoplasmic tyrosine kinase Abl in DCR formation (Plattner et al., 1999). Both, PKA and Abl, are known to be involved in EMT (Srinivasan and Plattner, 2006; Yang et al., 2006; Finn et al., 2007). β-catenin in a complex with APC is also found to be localized at the leading edge of migratory cells, implying an important role for cell polarization and migration by linking microtubules to the actin cytoskeleton (Etienne-

Manneville and Hall, 2003; Watanabe et al., 2004; Sharma et al., 2006). Notably, β-catenin seems to exert its function at the leading edge of cells by co-localizing with N-cadherin and IQGAP1 in membrane ruffles (Sharma and Henderson, 2007). IQGAP1, an effector of Cdc42 and Rac1, here acts as a key regulator of internalization of N-cadherin and APC. The fact that PDGFR also co-localizes with N-cadherin in membrane ruffles suggests an interesting overlap of proteins known to be important in both EMT and ruffle or podosome formation (Theisen et al., 2007). Other newly identified players in DCR formation and important for motility are palladin and its interaction partner Eps8 (Goicoechea et al., 2006; Goicoechea et al., 2008). Palladin binds F-actin and crosslinks actin filaments into bundles. It also interacts with Src, thereby affecting Src-mediated actin remodeling (Ronty et al., 2007). Both proteins are also involved in podosome formation which highlights the kinship between DCRs and podosomes (Goicoechea et al., 2008). The cancer relevance of Eps8 and palladin is underlined by their increased expression in various human cancers, including breast and thyroid cancer (Matoskova et al., 1995; Ryu et al., 2001; Wang et al., 2004; Griffith et al., 2006; Yao et al., 2006). Interestingly, palladin expression appears to be induced by treatment of myofibroblasts with TGFβ1, suggesting a link between TGFβ signaling, a major inducer of EMT, and the formation of ruffles and podosomes (Ronty et al., 2006).

1.4.9. Lamellipodia and filopodia

To be able to leave the primary tumor and to disseminate to secondary sites, tumor cells have to break down cell-cell and cell-matrix junctions, as described above, and they have to invade into the surrounding tissue by gaining motility and forming invasive, matrix degrading structures. On planar two-dimensional culture substrates, cells utilize two well-known organelles to explore and move into the surrounding environment, filopodia and lamellipodia. Both are actin-rich membrane protrusions that are formed upon remodeling of the actin cytoskeleton beneath the plasma membrane. Lamellipodia are flat, sheet-like protrusion and they are the main organelle for cell locomotion. The unbranched, long actin filaments at the base of the lamellipodium are progressing into a highly, lateral branched actin network at the leading edge, giving lamellipodia their typical structure (Figure 5B). Here, actin assembly

and lateral branching (dendritic nucleation) are mainly controlled by the Arp2/3 complex, a seven subunit protein and major initiator of actin assembly. The activity of the Arp2/3 complex itself is controlled by actin nucleation-promoting factors, such as the N-WASP or the Scar/WAVE complexes which are themselves recruited to and activated at the membrane by Rac1 (Innocenti et al., 2004; Ibarra et al., 2005; Le Clainche and Carlier, 2008). Increased expression of Arp2/3 and WAVE2 has been shown to correlate with poor prognosis in breast and liver carcinomas underlining the relevance of lamellipodia-like structures in cancer progression (Iwaya et al., 2007a; Iwaya et al., 2007b). Furthermore, the formation of lamellipodia is also observed upon ErbB2-driven EMT in epithelial cells, indicating that the formation of lamellipodia-like structures underlies the increased invasiveness observed during EMT (Khoury et al., 2001). Lamellipodia interact and attach to their environment via different adhesion molecules, including integrins and cadherins (Comunale et al., 2007; Wang et al., 2008). Also, CD44, the hyaluronan receptor was identified to be in a complex with the protease MT1-MMP in lamellipodial protrusions (Mori et al., 2002). Here, MT1-MMP binds directly to CD44 and mediates its proteolytic cleavage, thereby stimulating migration (Figure 5B). In contrast, filopodia are rod-like extension consisting of tight bundled actin fibers which penetrate into the surrounding environment originating from the basis of lamellipodia. Filopodia can be considered as a sensory organ of the cell that are used to detect and assimilate signals like chemoattractants or nutrients released from e.g. blood vessels. Interestingly, metastatic cells are rich in filopodia-like structures, which correlates with their invasiveness (Coopman et al., 1998; Wang et al., 2002). Filopodia formation is based on non-branched, processive actin assembly, controlled by fascin, diaphanous and Ena/ VASP (Svitkina et al., 2003; Le Clainche and Carlier, 2008) (Figure 5B). Actin bundling via fascin, diaphanous and Ena/VASP, on the other hand, is controlled by Rac and Cdc42. Fascin upregulation correlates with poor prognosis in different cancer types, including gastric cancer, lung cancer and breast cancer (Pelosi et al., 2003; Hashimoto et al., 2004; Rodriguez-Pinilla et al., 2006). Notably, fascin is a direct target the β-catenin/Tcf signaling pathway, which is active in the process of EMT as well (Vignjevic et al., 2007). Recently, it has been reported that filopodia can transform into lamellipodia by initiating dendritic actin nucleation,

characteristic for lamellipodia formation, demonstrating that both filopodia and lamellipodia are highly interactive, inter-convertible structures (Mongiu et al., 2007).

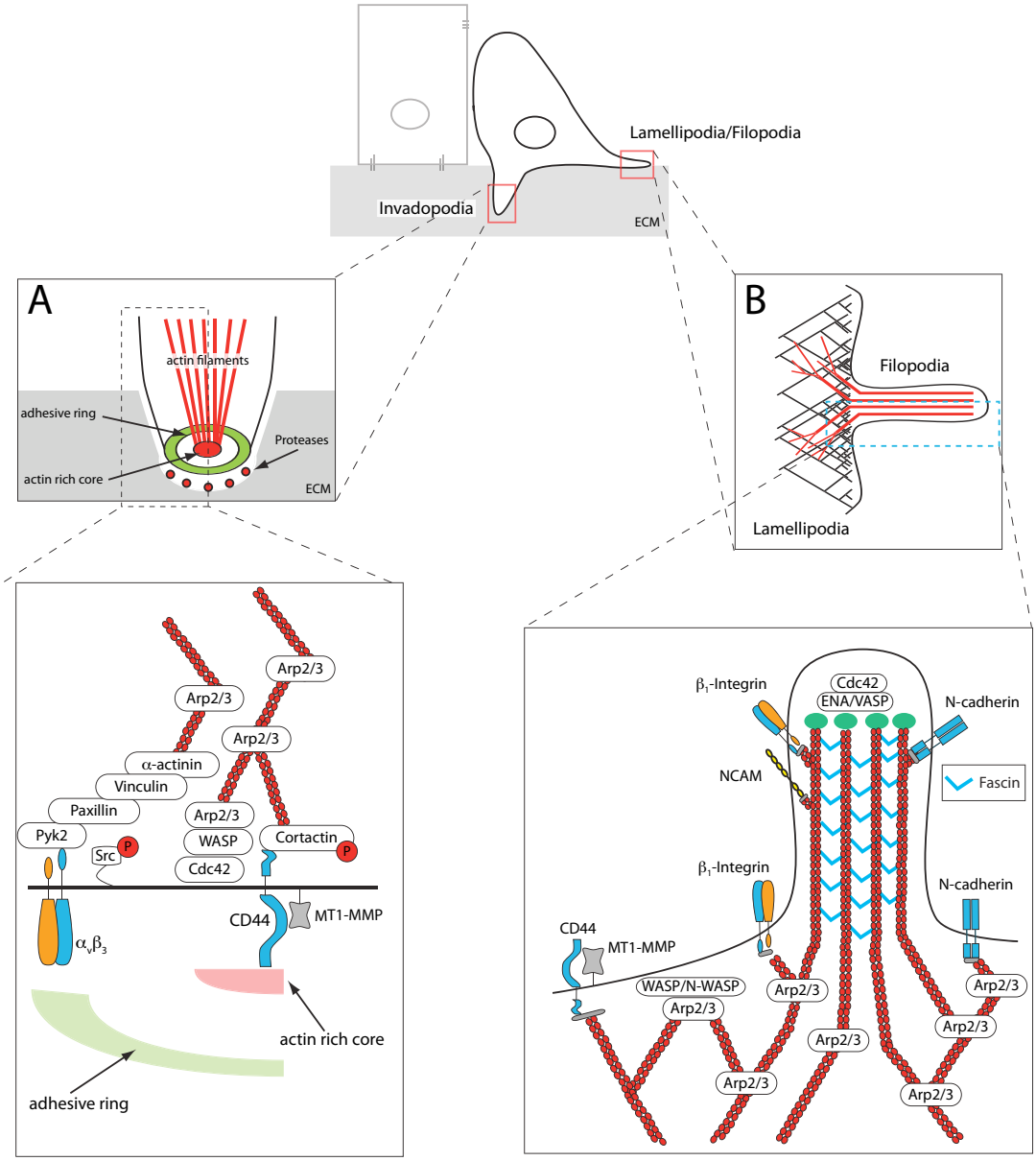


Figure 5. Upon completion of EMT, spindle-shaped, invasive cells migrate and invade into the surrounding environment by utilizing (i) membrane protrusion like lamellipodia and filopodia for horizontal cell movement and (ii) podosome-derived invasive structures, invadopodia, for ventral invasion. **(A) Invadopodia-mediated invasion.** Invadopodia are actin and phosphotyrosine-rich membrane protrusions with a high extracellular matrix degrading capacity. Invadopodia consist of an outer adhesive ring and a central actin-rich core. The non-receptor tyrosine kinase Src, the actin binding protein cortactin and the membrane bound matrix metalloproteinase MT1-MMP play an essential role in the formation and function of invadopodia. Integrins (like $\alpha_v\beta_3$-integrin), along with their cytoplasmic interaction partners, are positioned in the adhesive ring where they mediate adhesion. The actin organization and nucleation is controlled by the Arp2/3 protein complex (adapted from Block et al., 2008). **(B) Lamellipodia/filopodia-mediated migration.** Lamellipodia are the major organelles for cell movement and build upon highly branched dendritic actin networks, which are initiated by the nucleation promoting

factors WASP/N-WASP and the Arp2/3 complex. Lamellipodia interact with their environment via N-cadherin, β_1-integrin or the hyaluronan receptor CD44, the latter also important for the correct positioning of the membrane-bound MT1-MMP. Filopodia are stiff, rod-like extension formed by tightly bundled actin filaments. They function as sensory organs of cells that penetrate into the surrounding microenvironment. They originate from the root of lamellipodia and are initiated and controlled by the concerted activity of the RhoGTPase Cdc42 and nucleation-promoting factor ENA/VASP. The major determinant for actin bundling and filopodia morphology is fascin. Filopodia interact with their environment via the cell adhesion molecules N-cadherin, NCAM, and β_1-integrins.

1.4.10. Podosomes and invadopodia

To move into or through a three-dimensional matrix, the real life situation, cells have to gain the capability to remodel the ECM via the expression of proteinases. In the last few years, actin-rich protrusion with ECM proteolytic activity, called invadopodia, have drawn particular attention in the context of cancer cell invasion. Invadopodia are considered the transformed counterparts of podosomes, which are formed by non-transformed yet highly migratory cells, such as macrophages, dendritic cells, osteoclasts, and activated smooth muscle and endothelial cells. The exact functions of podosomes are still rather elusive, yet it seems that they are formed when cell adhesion junctions and matrix needs to be concomitantly degraded. This may be the case, when immune cells like monocytes or dendritic cells have to cross tissue boundaries or during bone resorption by osteoclasts for the control of bone homeostasis (Saltel et al., 2004). The similarities or non-similarities of podosomes and invadopodia are still under debate, yet the most striking differences between both lies in the degree of ECM degradation and their half-lives. Invadopodia are strong degraders of ECM and persist for up to one hour, whereas podosomes exhibit less ECM degradation activity and are stable only for minutes. Common for invadopodia and podosomes is their architecture. They share an actin filament-rich core containing the actin assembly machinery (WASP, cortactin and Arp2/3) and a multimeric protein complex surrounding the actin core, consisting of integrins and integrin-associated proteins like vinculin, talin and paxillin (Figure 5A). The multimeric adhesive ring complex is connected to the actin core via radial actin filaments (Linder and Kopp, 2005; Ayala et al., 2006; Linder, 2007; Block et al., 2008). Invadopodia of invading tumors cells mediate proteolysis of the ECM via the expression of different MMP, the most prominent being MT1-MMP collagenase and one of its direct targets, MMP2 gelatinase (Kelly et al., 1998). Invadopodia have been

identified in numerous cancer cell lines, including malignant melanoma, breast cancer, glioma, and head and neck cancer (Angers-Loustau et al., 2004; Tague et al., 2004; Artym et al., 2006; Clark et al., 2007). The formation of invadopodia can be initiated by various signals, for example by EGF, HGF or TGFβ-induced signal transduction (Tague et al., 2004; Yamaguchi et al., 2005; Oxmann et al., 2008) or by $\alpha_6\beta_1$-integrin engagement (Nakahara et al., 1996). Invadopodia-inducing growth factors are not only produced by cancer cells themselves but are frequently supplied by tumor-associated macrophages (TAM), which themselves are attracted to the tumor microenvironment by tumor-released chemokines, such as colony stimulating factor 1 (CSF-1) or placental growth factor (PlGF). TAM are well-known to increase cancer cell invasion for example by inducing EGF driven invadopodia formation in the cancer cells (Wyckoff et al., 2004; Yamaguchi et al., 2006). Besides their important role in primary tumor invasion and tumor cell intravasation into the blood or the lymphatic circulation, invadopodia are also critical for extravasation at secondary sites. Thereby, the chemokines S100A8 and S100A9, produced by endothelial or myeloid cells at the premetastatic niche, attract circulating tumor cells and facilitate their extravasation and invasion at the secondary site by inducing the formation of tumor cell invadopodia (Rafii and Lyden, 2006). The formation of invadopodia follows distinct steps (Artym et al., 2006). First, a local enrichment of actin and cortactin at sites of cell-ECM contact initiates the recruitment of MT1-MMP. This stage is named the pre-invadopodia stage and is then followed by further recruitment of actin, cortactin and MT1-MMP, which leads to matrix degradation and the formation of mature, invading invadopodia. Late invadopodia are defined by dispersal of actin and cortactin leaving an MT1-MMP enriched structure that maintains matrix degradation. A recent report proposes important roles for the calcium-dependent protease calpain-2, protein tyrosine phosphatase PTP1B, and Src for the onset of invadopodia formation and the regulation of invadopodia turnover (Cortesio et al., 2008). Apparently, activation of integrins and/or EGF receptor leads to increased calpain-2 activity, which subsequently proteolytically processes and activates PTP1B. Activated PTP1B wields dephosphorylation of an inhibitory phospho-tyrosine of Src resulting in increased Src tyrosine kinase activity, Src-mediated phosphorylation of cortactin and, subsequently, to actin

assembly. Conversely, activated Src phosphorylates calpain-2, resulting in proteolytic cleavage and inactivation of cortactin and, thus, invadopodia disassembly. Both Src and cortactin are essential for invadopodia formation and their increased activity can be used as a potential marker for the onset of invadopodia formation (Bowden et al., 2006; Webb et al., 2007). Downstream targets of activated Src which are known to be essential for invadopodia formation and maintenance include cortactin, N-WASP, ArfGAPs like AMAP1/AMAP2, paxillin and Tks5/Fish (Seals et al., 2005; Yamaguchi et al., 2005; Bharti et al., 2007; Badowski et al., 2008; Oikawa et al., 2008). Invadopodia are known to be enriched with integrins of the β_1-integrin family (α_3, α_5, α_6) or with $\alpha_v\beta_3$-integrin (Mueller and Chen, 1991; Deryugina et al., 2001; Badowski et al., 2008). β_1-integrins are receptors for laminin, collagen and fibronectin, $\alpha_v\beta_3$-integrins preferentially bind to vitronectin. Furthermore, the interaction of $\alpha_v\beta_3$-integrin with MT1-MMP leads to the activation of immature MMP2, thereby promoting collagen proteolysis in invasive breast carcinomas (Deryugina et al., 2001). Each of both proteins, $\alpha_v\beta_3$-integrin as well as MT1-MMP, have been shown to be important for EMT (Pulyaeva et al., 1997; Galliher and Schiemann, 2007). The co-expression and most likely co-operation of MT1-MMP and β_1-integrin in EMT-driven invasiveness, under the control of the transcription factor Twist, suggests that proteins relevant for invadopodia formation are important for EMT as well (Terauchi et al., 2007). Besides their function as mechanical anchors in the ECM, integrins actively participate in invadopodia formation. For example, engaged $\alpha_6\beta_1$-integrin induces phosphorylation of p190RhoGAP and promotes its localization in invadopodia, where it represses RhoA activity and supports the formation of membrane protrusions (Nakahara et al., 1998). As key regulators of actin assembly, the major RhoGTPases RhoA, Rac1, and Cdc42 play essential roles in invadopodia formation. Elimination of the activities of Rac1 or Cdc42 results in a reduction of invadopodia formation in glioma cells and mammary carcinoma cells, respectively (Chuang et al., 2004; Yamaguchi et al., 2005). On the other hand, RhoA is known to be important for actin assembly and exocytosis of MMP in invadopodia by controlling IQGAP1 activity (Sakurai-Yageta et al., 2008). For more detailed insights into invadopodia formation and function, we refer the reader to several excellent recent reviews (Buccione et al., 2004;

Linder, 2007; Gimona et al., 2008; Vignjevic and Montagnac, 2008).

1.4.11. Lamellipodia, filopodia and their overlap with invadopodia

The kinship between the two-dimensional filopodia and lamellipodia with three-dimensional invadopodia is still under debate. It seems that invadopodia represent a hybrid of both structures. Like lamellipodia, invadopodia are built upon a branched actin network, yet they employ N-WASP for actin assembly instead of WAVE-2 used by lamellipodia. On the other hand, both invadopodia and filopodia engage Cdc42 for the induction of membrane protrusions which are long and thin in their appearance, suggesting that actin bundling could play an important role in invadopodia formation. However, whether filopodial actin-bundling proteins like fascin are located in invadopodia remains to be determined (Weaver, 2008).

1.4.12. Invadopodia and their role in EMT

As illustrated above, EMT leads to increased motility and invasiveness of epithelial cells by dissolving epithelial cell-cell adhesion, modulating cell-matrix adhesion and by inducing the secretion of ECM degrading proteinases. Thus, it can be assumed that EMT provokes the formation of podosome or invadopodia-like structures. Surprisingly, up to date there is no experimental evidence that directly links EMT with the formation of invadopodia or podosomes. Nonetheless, a number of proteins that are important for invadopodia formation and for the execution of EMT have been identified, strongly suggesting that invadopodia formation could be involved in EMT-driven cell invasion. Both EMT and invadopodia formation can be stimulated by various growth factors, including EGF, HGF, PDGF, and TGFβ (Yamaguchi et al., 2005; Lo et al., 2007), (Varon et al., 2006). The engagement of these growth factors leads to the activation of the non-receptor tyrosine kinase Src. The potency of Src to induce EMT and/or invadopodia formation is well established (Frame, 2004; Bowden et al., 2006; Cortesio et al., 2008). Moreover, downstream targets of Src-signaling, such as calpain, are upregulated during TGFβ-induced EMT and are required for invadopodia formation (Xie et al., 2003; Cortesio et al., 2008). Another protein, able to induce invadopodia and also involved in EMT, is endoglin, a cell surface adhesion molecule

and co-receptor for TGFβ signaling (Fonsatti et al., 2003). Endoglin is needed for EMT during heart development, and its expression correlates with increased invasiveness of breast cancer cells via the increased formation of invadopodia (Mercado-Pimentel et al., 2007; Oxmann et al., 2008). Most likely, invadopodia and podosomes are not only matrix degrading structures but also function as guidance organelles that provide a cell with the ability to probe its microenvironment by exploring potential cell-cell and cell-matrix adhesions and by sensing chemoattractive cues. Interestingly, PDGF induces the translocation of cortactin to podosomes via the GAP BPGAP1 and Rac1, indicating that PDGF receptor is involved in the formation of podosomes (Head et al., 2003; Lua and Low, 2004). As described above, PDGFR colocalizes with N-cadherin in membrane ruffles, and both N-cadherin and cortactin are found in the growth cones of emerging neuronal axons and exert a crucial role in neuron guidance (Lee, 2005; Bard et al., 2008). The co-localization of cortactin with the N-cadherin/ NCAM/FGFR complex and NCAM-mediated FGFR signaling have been shown to contribute to neurite outgrowth (Cavallaro et al., 2001). Thus, consistent with its upregulated expression during EMT (the cadherin switch, see above), N-cadherin is likely to be involved in the formation of invadopodia.

2. AIM OF THE STUDY

Epithelial-mesenchymal transition (EMT) is a critical process underlying the onset of cancer cell invasion and has been identified at the invasive front of murine and human tumors. Hence, the identification of reliable prognostic markers and the intervention with EMT-driven invasion is of major interest in cancer diagnostics and therapy. To achieve these goals, we need to gain new and detailed insights into the molecular processes regulating EMT.
For this purpose, we induced EMT in different *in vitro* model systems and followed changes in gene expression profile to investigate:

- which genes are master regulatory genes of EMT
- which cell biological processes are controlled by these master regulators
- are these master regulators required for tumor progression and metastasis formation *in vivo*
- are these master regulators potential markers for invasion and metastasis in human tumors

Here, we identified the cell adhesion molecule NCAM and the transcription factor Dlx2 as master regulatory genes of EMT and investigated there cell biological functions as well as their contribution to tumor progression and metastasis formation *in vivo*.

3. RESULTS

3.1. NCAM-induced focal adhesion assembly: a functional switch upon loss of E-cadherin (Article)

Francois Lehembre, Mahmut Yilmaz, Andreas Wicki, Tibor Schomber, Karin Strittmatter, Dominik Ziegler, Angelika Kren, Phillip Went, Patrick WB Derksen, Anton Berns, Jos Jonkers and Gerhard Christofori. EMBO 2008, 27(19), 2603-15.

3.1.1. Abstract

Loss of expression of the cell-cell adhesion molecule E-cadherin is a hallmark of epithelial-mesenchymal-transition (EMT) in development and in the progression from epithelial tumors to invasive and metastatic cancers. Here, we demonstrate that the loss of E-cadherin function up-regulates expression of the neuronal cell adhesion molecule NCAM. Subsequently, a subset of NCAM translocates from fibroblast growth factor receptor (FGFR) complexes outside of lipid rafts into lipid rafts where it stimulates the non-receptor tyrosine kinase $p59^{Fyn}$ leading to the phosphorylation and activation of focal adhesion kinase (FAK) and the assembly of integrin-mediated focal adhesions. Ablation of NCAM expression during EMT inhibits focal adhesion assembly, cell spreading and EMT. Conversely, forced expression of NCAM induces epithelial cell delamination and migration, and high NCAM expression correlates with tumor invasion. These results establish a mechanistic link between the loss of E-cadherin expression, NCAM function, focal adhesion assembly and cell migration and invasion.

3.1.2. Introduction

When cancer cells disseminate from a primary tumor and infiltrate the surrounding tissue, they leave the tumor mass not only by gaining migratory and invasive capabilities but also by dissolving cell-cell contacts, known as tight and adherens junctions (Friedl and Wolf, 2003b). Consistent with this notion, the calcium-dependent cell-cell adhesion molecule E-cadherin, the prototype adhesion molecule of epithelial adherens junctions, is found absent or

dysfunctional in the majority of epithelial cancers (carcinomas) (Cavallaro and Christofori, 2004). Inhibition of E-cadherin activity by specific neutralizing antibodies or shRNA-mediated down-regulation of its expression in epithelial MDCK cells leads to increased motility and invasiveness (Behrens et al., 1989; Qin et al., 2005). Conversely, the invasive behavior of carcinoma cells can be blocked by forced expression of E-cadherin (Frixen et al., 1991). Using a transgenic mouse model of pancreatic β cell carcinogenesis (Rip1Tag2), we have previously demonstrated that the loss of E-cadherin is a rate-limiting event during tumor progression (Perl et al., 1998).

In many cancer types, the loss of E-cadherin coincides with a gain of expression of the mesenchymal cadherin, N-cadherin. This 'cadherin switch' is thought to be required for tumor cells to gain invasive properties (Cavallaro, 2004). Interestingly, the cadherin switch is also a hallmark of EMT, a process characterized by the combined loss of epithelial cell junction proteins, including E-cadherin, α-catenin, β-catenin, and claudins, and the gain of mesenchymal markers, such as N-cadherin, vimentin and fibronectin (Thiery, 2003; Thiery and Sleeman, 2006). EMT is a critical process during embryonic development and it is thought to play an important role in the progression of cancer, although this hypothesis is still under debate (Lee et al., 2006).

Here, we have investigated the functional consequences of the loss of E-cadherin in various cellular systems and transgenic mouse models and report that expression of the neuronal cell adhesion molecule NCAM, a member of the immunoglobulin superfamily of genes, is up-regulated concomitant with the loss of E-cadherin function. NCAM has been previously implicated in tumor progression and in lymph node metastasis, mainly by modulating β_1 integrin-mediated cell-matrix adhesion (Cavallaro et al., 2001; Christofori, 2003). In neurons, NCAM triggers distinct signal transduction pathways. Within lipid rafts, NCAM associates with and activates $p59^{Fyn}$ kinase leading to the phosphorylation of focal adhesion kinase (FAK) and focal adhesion assembly, whereas in the non-raft compartment NCAM facilitates FGFR–activated signaling (Beggs et al., 1997; Niethammer et al., 2002). Here, we demonstrate that the up-regulation of NCAM expression is a direct result of the loss of adherens junctions. As a consequence, NCAM clusters in lipid rafts together with $p59^{Fyn}$,

leading to FAK phosphorylation and focal adhesions assembly, activities required for cell motility and EMT.

3.1.3. Results

3.1.3.1. EMT induced by the loss of E-cadherin

We have employed several different experimental systems to investigate the functional impact of the loss of E-cadherin function on cell behavior and cell signaling. First, TGFβ-treatment of NMuMG murine mammary gland epithelial cells, a well-characterized EMT model, induced a loss of E-cadherin expression accompanied by a complete EMT (Miettinen et al., 1994; Bhowmick et al., 2001a) (Figure 6A). Second, shRNA-mediated knock-down of E-cadherin in MCF7 human epithelial breast carcinoma cells substantially reduced E-cadherin expression (MCF7-shEcad; Figure 6A,B). Third, genetic ablation of E-cadherin function was achieved by establishing a cell line from mammary tumors of MMTV-Neu transgenic mice (Muller et al., 1988), in which both E-cadherin alleles were flanked by LoxP recombination sites (MTflEcad cells) (Derksen et al., 2006). Expression of Cre-recombinase in MTflEcad cells led to a complete abrogation of E-cadherin expression (MTdeltaEcad; Figure 6A,B). The lack of E-cadherin expression in both MCF7-shEcad and MTflEcad cells resulted in a loss of epithelial morphology and a gain of a fibroblast-like appearance (Figure 6A). Reduced expression of E-cadherin in MCF7 cells was accompanied by decreased expression of other epithelial markers, such as β−catenin, and an increased expression of the mesenchymal markers N-cadherin and vimentin. The lack of E-cadherin in MTflEcad cells only induced N-cadherin expression, without any up-regulation of vimentin or loss of β−catenin (Figure 6B). Furthermore, MCF7-shECad cells exhibited a 8-fold increase in cell motility and a 3-fold increase in invasiveness, whereas MTdeltaEcad cells only displayed a 2.5-fold elevated motility but no increased invasiveness (Supplementary Figure S1A,B). These data indicate that removal of E-cadherin can induce either a *bona fide* EMT (MCF7-shEcad) or merely a “cadherin-switch” in the absence of a full EMT (MTdeltaEcad). Notably, cell proliferation was diminished upon E-cadherin-depletion in all experimental systems tested (Supplementary Figure S1C).

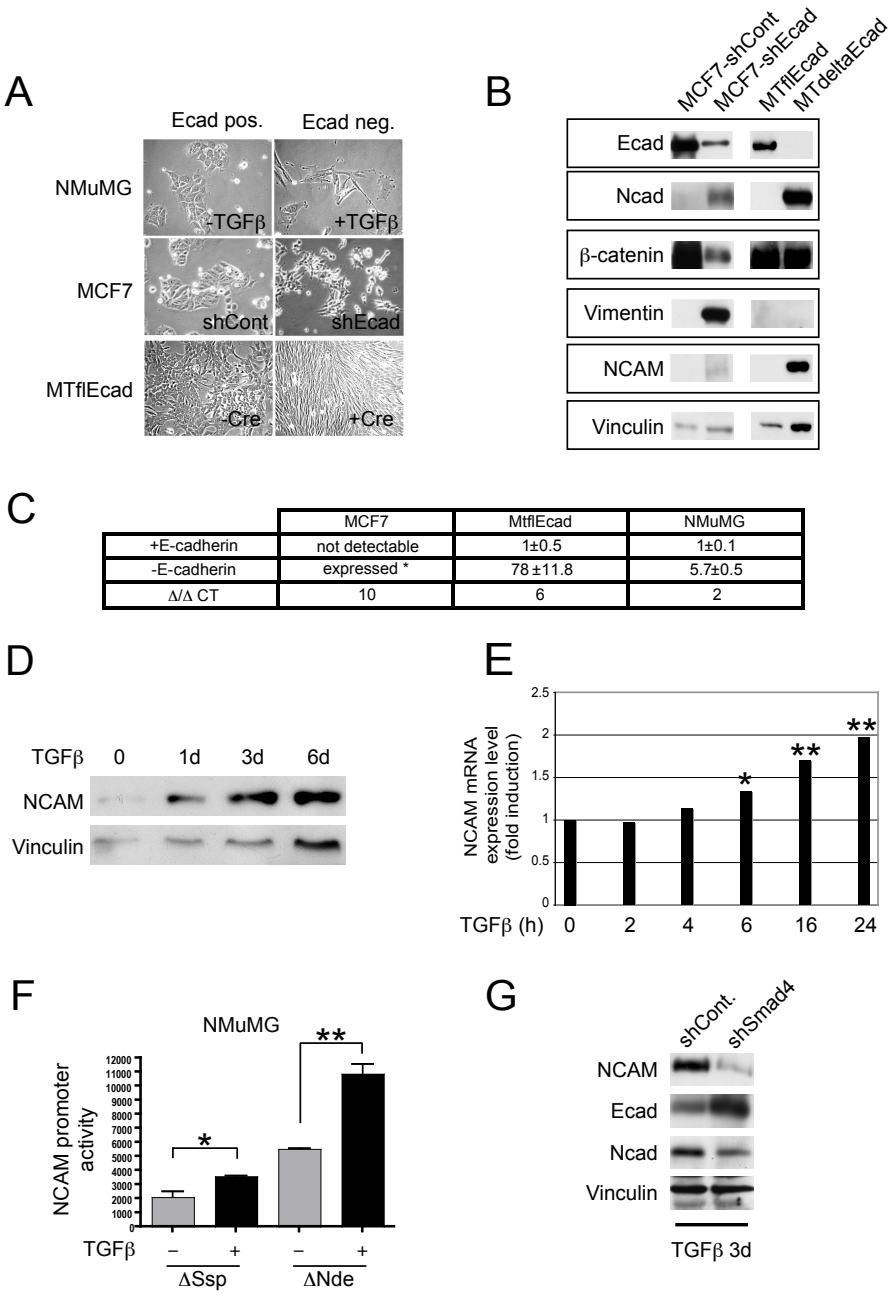


Figure 6. E-cadherin loss in epithelial cell lines induces either a cadherin switch or full EMT.**(A)** NMuMG, MCF7 and MTflEcad cells adapt a mesenchymal phenotype upon loss of E-cadherin by TGFβ treatment (+TGFβ), shRNA expression (shEcad) or Cre-mediated deletion of the *E-cadherin* gene (+Cre). **(B)** Immunoblot analysis for E-cadherin (Ecad), N-cadherin (Ncad), β-catenin, vimentin and NCAM expression in MCF7 and MTflEcad cells expressing E-cadherin (shCont and MTflEcad) or depleted for E-cadherin expression by shRNA expression (shEcad) or Cre-mediated deletion of the E-cadherin locus (MTdeltaEcad). Cell extracts were loaded on different gels and resolved proteins were visualized with specific antibodies as indicated. Immunoblotting for vinculin was used as loading control. **(C)** NCAM expression is induced

upon loss of E-cadherin function. NCAM mRNA levels were determined by quantitative RT-PCR in MCF7, MTflEcad and NMuMG cells that have been depleted for E-cadherin (-E-cadherin) or not (+E-cadherin). For all values $p<0.0001$, unpaired t-test. Data are shown as mean±SD. *Since NCAM is not expressed at detectable levels in MCF7 cells, its upregulated expression in MCF-shEcad cells cannot be calculated as fold-induction. Instead the comparative Ct values (ΔΔCt) are given in the lower row. **(D)** Immunoblot analysis for NCAM protein expression in NMuMG cells in the presence of TGFβ at the indicated time points. Immunoblotting for vinculin was used as loading control. **(E)** NCAM mRNA levels were determined by quantitative RT-PCR in NMuMG cells treated with TGFβ for the times indicated. *$p=0.0134$, **$p<0.005$; unpaired t-test). Data are shown as mean±SD. **(F)** NCAM promoter activity is increased upon loss of E-cadherin expression. NMuMG were transfected with a CAT reporter plasmid containing 1000 bp (ΔSsp) or 647 bp (ΔNde) of the NCAM promoter sequence and treated or not with TGFβ for 6 days prior to the analysis of CAT activity (left panel; *$p=0.0027$ and **$p=0.0007$, unpaired t-test). Data are shown as mean±SD. **(G)** NMuMG cells stably transfected with shControl or shSmad4 were treated for 3 days with TGFβ and then analyzed for NCAM, E-cadherin (Ecad) and N-cadherin (Ncad) expression by immunoblotting analysis. Immunoblotting for vinculin was used as loading control.

3.1.3.2. EMT induces NCAM expression

Gene expression profiling experiments and quantitative RT-PCR analysis comparing NMuMG, MCF7 and MTflEcad cells before and after the loss of E-cadherin function revealed that the expression of NCAM was significantly up-regulated upon loss of E-cadherin expression in TGFβ-treated NMuMG, MCF7-shEcad and MTdeltaEcad cells (Figure 6C). Immunoblotting analysis confirmed up-regulated expression of the 140 kD isoform of NCAM (NCAM140) in comparison to the E-cadherin-expressing cells (Figure 6B). NCAM mRNA up-regulation was already detected 6 hours after TGFβ treatment of NMuMG cells, and an increase in NCAM protein levels was observed 24 hours after TGFβ treatment (Figure 6D,E), indicating that the upregulation of NCAM expression is an early event upon loss of E-cadherin function and EMT. Ablation of E-cadherin by treatment of MTflEcad cells with recombinant Cre recombinase protein carrying a HIV-Tat cell entry-peptide resulted in the concomitant loss of E-cadherin expression with the gain of NCAM expression, demonstrating that NCAM expression is upregulated by the loss of E-cadherin without any additional TGFβ stimulation (Supplementary Figure S2B). Also in HEK293 cells, mesenchymal phenotype cells that expressed low levels of E-cadherin, shRNA-mediated ablation of E-cadherin expression resulted in an elongated cell shape with increased motility and an up-regulation of NCAM expression (Supplementary Figure S3A-C). Next, we investigated the effect of loss of E-cadherin expression on *NCAM* gene promoter activity by CAT-reporter assays. Utilizing DNA sequences of the *NCAM* gene promoter region either 1

kb (ΔSsp) or 647 bp (ΔNdeI) upstream of the transcriptional start site (Boras and Hamel, 2002), TGFβ-treatment of NMuMG cells (Figure 6F) or ablation of E-cadherin expression in MCF7 cells (Supplementary Figure S2A) provoked a significant increase in NCAM promoter-reporter activity. It has been recently reported that Smad4-depleted NMuMG cells (NMuMG-shSmad4) maintain an epithelial morphology upon TGFβ treatment (Deckers et al., 2006). Consistent with this report, NMuMG-shSmad4 cells still expressed high levels of E-cadherin after 3 days of TGFβ treatment, but only low levels of NCAM and N-cadherin in comparison to Smad4-expressing NMuMG-shControl cells (Figure 6G). These results indicate that Smad4-mediated signal transduction plays a critical role in the induction of *NCAM* gene expression during TGFβ-induced EMT. To investigate whether NCAM up-regulation also occurs *in vivo,* we crossed mice carrying LoxP-flanked conditional alleles of the E-cadherin gene (Derksen et al., 2006) with Rip1Cre mice in which Cre-recombinase was specifically expressed in β cells of the pancreatic islets of Langerhans (Ahlgren et al., 1998). Despite the low constitutive expression of NCAM in islet cells of normal control mice (Esni et al., 1999), β cells that upon successful recombination had lost E-cadherin expression exhibited significantly higher levels of NCAM at their cell surface (Supplementary Figure S4). Together, these data indicate that the loss of E-cadherin causes increased NCAM gene expression *in vivo* and *in vitro*.

3.1.3.3. NCAM is required for EMT

We next assessed whether increased NCAM levels were required for epithelial cells to undergo EMT. Three murine NCAM-specific siRNAs (simNCAM1, 2 and 3) efficiently down-regulated NCAM mRNA and protein levels in NMuMG cells in comparison to control NCAM-mismatch-siRNA (siControl) transfected cells (Figure 7A,B). NCAM-depleted NMuMG cells failed to undergo EMT and to upregulate the mesenchymal marker N-cadherin upon TGFβ treatment, while siControl-transfected NMuMG cells readily converted to mesenchymal cells indicating that NCAM is required for EMT to occur (Figure 7A,C). Notably, ablation of NCAM expression in NMuMG cells, that after 13 days of TGFβ treatment had undergone full EMT, lead to a reversion of EMT, with characteristic polarized epithelial cell morphology. This morphological change was accompanied by an increase in E-

cadherin expression and a decrease in N-cadherin expression, demonstrating that NCAM is not only required for the initiation but also for the maintenance of EMT in NMuMG cells (Figure 7D).

To investigate the role of NCAM in tumors undergoing EMT *in vivo*, we analyzed E-cadherin expression during tumor progression in the Rip1Tag2 (RT2) transgenic mouse model of pancreatic β cell carcinogenesis (Hanahan, 1985), either wildtype for NCAM expression or in a NCAM knock-out background (RT2;NCAM+/+ and RT2;NCAM-/-, respectively; (Perl et al., 1999). In wildtype RT2, the tumor areas showing downregulation of E-cadherin expression exhibited significantly higher intensities of diffuse NCAM-specific fluorescence staining at cell-cell contacts (Figure 7E). In contrast, in the absence of NCAM expression, 75% of the invasive carcinomas of RT2;NCAM-/- mice showed high levels of E-cadherin expression (Figure 7F; $p<0.0001$, Fisher's exact test). Altogether, these results indicate that ablation of NCAM expression prevents EMT and E-cadherin loss *in vitro* and *in vivo*.

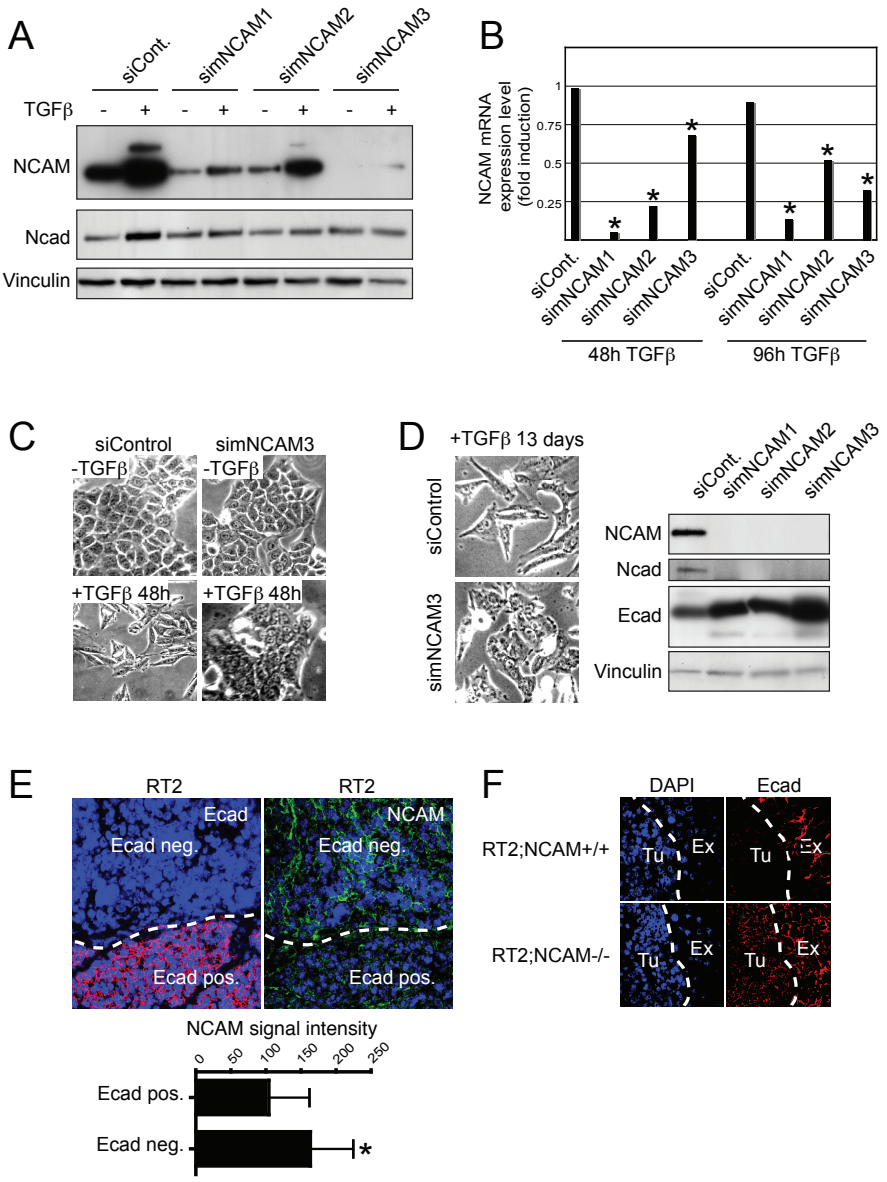


Figure 7. NCAM is required for EMT. **(A)** Immunoblot analysis of NCAM and N-cadherin (Ncad) in siControl and siNCAM-transfected NMuMG cells. 24 hours after transfection, TGFβ was added to the medium for 48 hours. Immunoblotting for vinculin was used as loading control. **(B)** Levels of NCAM mRNA were determined by quantitative RT-PCR in NMuMG cells that have been transfected with siControl and siNCAM for 24 hours before treatment with TGFβ for 48 or 96 hours. For all values *p<0.002 compared to siControl-transfected cells, unpaired t-test. Data are shown as mean±SD. **(C)** Phase contrast microscopy of siControl and siNCAM-transfected NMuMG cells treated or not with TGFβ for 48 hours. **(D)** NMuMG cells were treated for 13 days with TGFβ before transfection of a control (siControl) and or an NCAM-specific siRNA (siNCAM3). 48 hours later phase-contrast images were taken (left panels). NCAM, N-cadherin (Ncad) and E-cadherin (Ecad) expression levels were analyzed by immunoblotting (right panel). Antibodies against vinculin were used as loading control. **(E)** Immunofluorescence staining for E-cadherin (red) and NCAM (green) on pancreatic

sections from Rip1Tag2 mice. Lower panel: quantification of NCAM fluorescence in cell-cell junctions from E-cadherin-positive cells or E-cadherin-negative cells (each n = 40) shows increased expression in cells that have lost E-cadherin (*$p<0.0001$, unpaired t-test). Blue DAPI staining visualizes nuclei. **(F)** Immunofluorescence staining for E-cadherin (red) on pancreatic sections from Rip1Tag2;NCAM+/+ (upper panels), Rip1Tag2;NCAM-/- (lower panels) and Rip1Tag2 mice (right panels). Note that in contrast to carcinomas of Rip1Tag2;NCAM+/+ mice, E-cadherin expression is maintained in carcinomas of Rip1Tag2;NCAM-/- mice. Blue DAPI staining visualizes nuclei. Ex, exocrine tissue; Tu, tumor tissue.

3.1.3.4. NCAM is sufficient to induce hallmarks of EMT

We next examined whether NCAM was able to induce EMT in epithelial cells. Ectopic expression of NCAM induced cell death in NMuMG as well as in MCF7 cells, as previously reported with other EMT regulators (Mani et al., 2007). We thus established several independent clones of MDCK epithelial cells expressing NCAM140 (Figure 8A). Notably, NCAM-expressing MDCK cells formed numerous foci on top of the confluent cell monolayer, a feature that was not observed in control-transfected cells (Figure 8B). Under sparse conditions, NCAM-expressing cells scattered and did not form cell-cell contacts, while control MDCK cells formed small cell clusters (Figure 8B).

In heterogeneous populations of NCAM-expressing and non-expressing cells, the majority of the NCAM-expressing cells delaminated from the cellular monolayer and migrated on top of neighboring epithelial cells (Figure 8C). In the NCAM-expressing cells, adherens junction proteins, such as β-catenin and E-cadherin, were undetectable (Figure 8C), while N-cadherin expression was increased (Figure 8D). These results indicate that NCAM is able to induce morphological and molecular changes representing typical hallmarks of EMT in MDCK cells (shown here) and also in E-cadherin transfected fibroblastoid L cells (Esni et al., 1999).

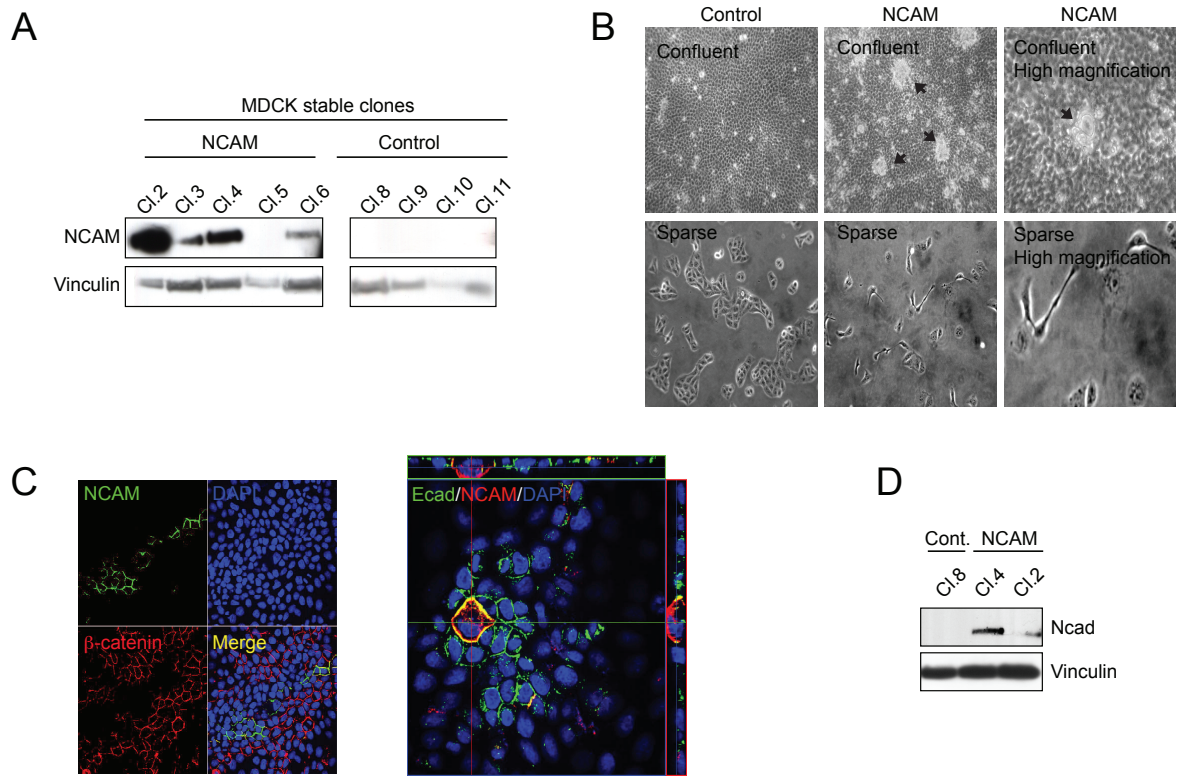


Figure 8. NCAM induces partial EMT. **(A)** Immunoblotting analysis of NCAM expression in MDCK cell clones either transfected with empty vector (control) or with an NCAM expression vector (NCAM). Immunoblotting for vinculin was used as loading control. **(B)** Phase-contrast microphotographs of confluent (upper panels) and sparse (lower panels) MDCK clones expressing NCAM (NCAM) or not (Control). Arrows indicate foci of cells growing on top of the cell monolayer. **(C)** Confocal laser scanning microscopy analysis of NCAM, β-catenin and E-cadherin (Ecad) expression in a mixture of MDCK cells expressing NCAM or not. Note that the NCAM expressing cells (green) do not express β-catenin (red) on their cell surface (left panel). The right panel shows a single NCAM-expressing cell that has lost E-cadherin expression and delaminates and migrates on top of the confluent, E-cadherin-expressing cell monolayer, as illustrated by the z- axis above and on the right side of the panel. **(D)** Immunoblot analysis of N-cadherin (Ncad) in MDCK cells stably expressing NCAM. Immunoblotting against vinculin was used as loading control.

3.1.3.5. NCAM induces focal adhesion assembly

Since we previously had shown that NCAM was able to modulate β_1 integrin-mediated cell-matrix adhesion (Cavallaro et al., 2001), we compared control and NCAM-depleted cells for their ability to spread on fibronectin-coated culture dishes. Cell spreading was significantly reduced in TGFβ-treated NMuMG cells upon NCAM removal (Figure 9A,B). Furthermore, cell spreading of TGFβ-treated, "mesenchymal" NMuMG cells could be repressed by neutralizing antibodies against β_1-integrin (Figure 9A), while spreading of non-treated

epithelial NMuMG cells was unaffected (Figure 9B). Consistent with this notion, numerous focal contacts were observed at the ventral surface of TGFβ-treated NMuMG cells in comparison to their epithelial counterparts (Figure 9C), and cells undergoing EMT exhibited increased amounts of phosphorylated FAK and paxillin and enhanced cell spreading (Figure 9D). Such TGFβ-mediated focal adhesion assembly as well as FAK phosphorylation was not observed in NCAM-depleted NMuMG cells (Figure 9C,D). In a time course of mesenchymal cell spreading, FAK was constantly phosphorylated suggesting a high stability of the focal adhesions, whereas in NCAM-depleted cells, the levels of FAK phosphorylation oscillated, indicating a high turnover of focal adhesions (Figure 9E).

Transwell migration assays revealed a significantly higher chemotactic migration of TGFβ-treated, siNCAM-transfected NMuMG cells as compared to TGFβ-treated, siControl-transfected cells (Figure 9F). Moreover, NCAM-depleted TGFβ-treated NMuMG cells migrated significantly faster in scratch wounding assays than NCAM-expressing control cells (Supplementary Figure S5). However, adhesion assays performed to quantify stable matrix attachment of cells independent of their spreading capacity did not reveal any differences between NCAM-expressing and non-expressing cells (data not shown). These data suggest that NCAM plays a critical role in focal adhesion assembly and turnover, rather than in modulating the strength of cell matrix-adhesion.

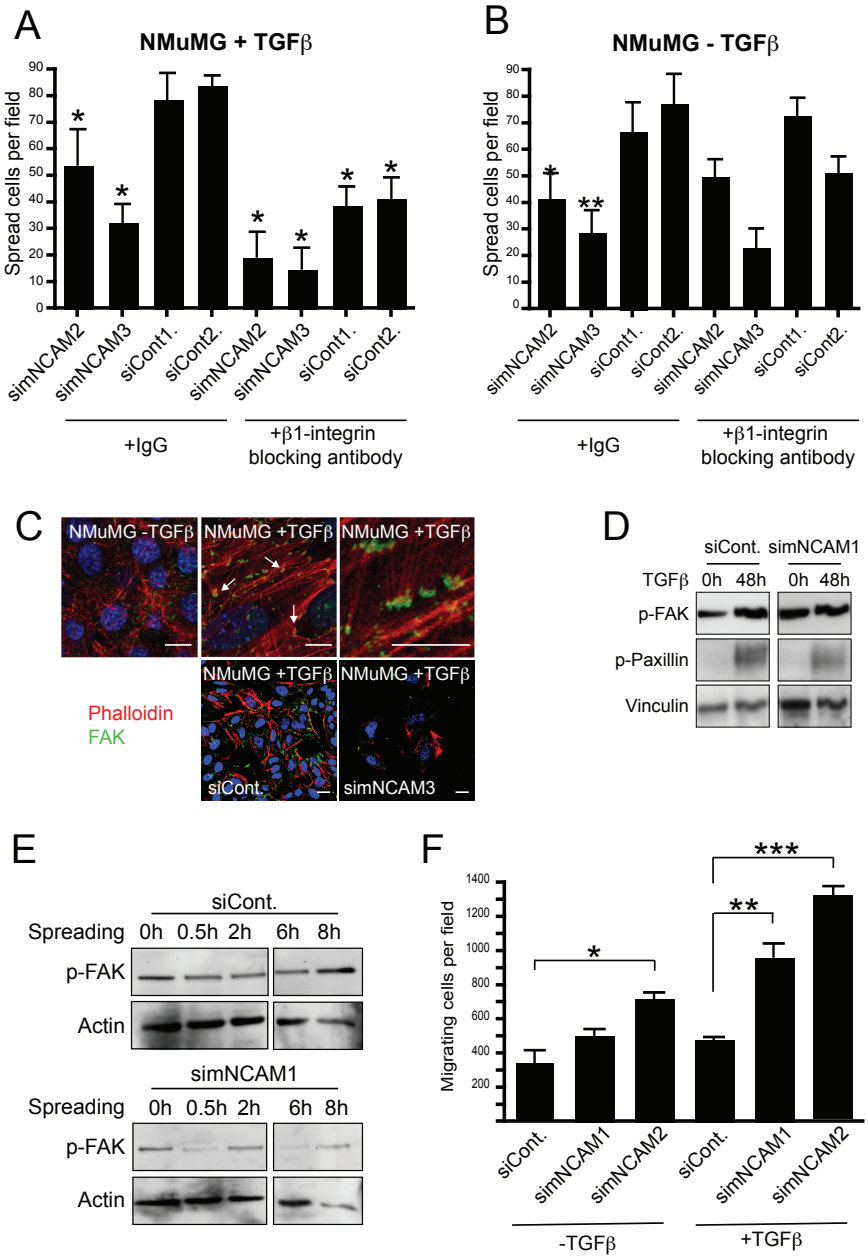


Figure 9. NCAM is required for focal adhesion assembly during EMT. **(A, B)** TGFβ-treated NMuMG cells show a markedly reduced cell spreading upon siRNA-mediated depletion of NCAM expression (simNCAM) as compared to control-treated cells (siCont). Cell spreading is further reduced by the addition of neutralizing antibodies against β_1-integrin in TGFβ-treated NMuMG cells (A) but not in untreated cells (B). Data are shown as mean±SD. *p< 0.05, unpaired t-test. **(C)** Immunofluorescence staining for actin (red) and $p125^{FAK}$ (green) in NMuMG cells in the presence or absence of TGFβ, as indicated. Numerous focal adhesions are detected in TGFβ-treated cells (arrows, middle upper panel) as compared to control cells (left panel). Higher magnification of the middle upper panel is shown on the right. Lower panels: NCAM-depletion leads to impaired focal adhesion assembly in siCont- and siNCAM-treated NMuMG cells treated with TGFβ.

Staining with DAPI (blue) visualizes nuclei. Size bars = 10 µm. **(D)** Immunoblotting analysis of NMuMG cells reveals an increase in FAK and Paxillin phosphorylation upon EMT induction (left panel). Treatment with siRNA against murine NCAM (simNCAM, right panel) represses the increase in EMT-induced FAK phosphorylation as observed in control-treated cells (siCont; left panel). Immunoblotting for vinculin was used as loading control. **(E)** TGFβ-treated NMuMG cells were transfected with control siRNA (siCont) or with siRNA targeting NCAM expression (simNCAM1), allowed to spread on the culture dish for the indicated time points and levels of FAK phosphorylation were determined by immunoblotting with antibodies specifically recognizing phosphorylated FAK. Immunoblotting for actin was used as loading control. **(F)** Depletion of NCAM expression (simNCAM)significantly induces migration of NMuMG cells trough Transwell filters as compared to control-siRNA-treated cells (siControl). *p=0.043, **p=0.013, ***p=0.0024, unpaired t-test. Data are shown as mean±SD.

3.1.3.6. NCAM re-localizes into lipid rafts upon EMT

In neurons, it has been reported that a signaling complex between NCAM, $p59^{Fyn}$ and FAK is formed upon homophilic NCAM-NCAM interactions, and that NCAM-mediated signaling via $p59^{Fyn}$ depends on its localization to lipid rafts (Beggs et al., 1997; Niethammer et al., 2002). Indeed, TGFβ treatment of NMuMG cells lead to increased phosphorylation of $p59^{Fyn}$ (Figure 10A). Immunofluorescence analysis revealed that in TGFβ-treated NMuMG cells**,** NCAM was predominantly localized in the junctions between cells and in focal plasma membrane areas (Figure 10B, upper panels) where it co-localized with FAK (Figure 10b, lower panels). Sucrose gradient density centrifugation experiments demonstrated that the low levels of NCAM present in epithelial NMuMG cells were found exclusively in the detergent-soluble protein fractions (Figure 10C). In contrast, in TGFβ-treated NMuMG cells a substantial proportion of the high levels of NCAM were also detected in the top fractions of the gradient (Figure 10C), indicating an association of NCAM with detergent-resistant lipid rafts. FAK and phosphorylated $p59^{Fyn}$ were also found in the NCAM-containing lipid raft fractions of the gradient. Disruption of lipid rafts by cholesterol depletion with methyl-β-cyclodextrin (M-β-CD) completely impaired the formation of NCAM clusters in TGFβ-treated NMuMG cells, while the actin cytoskeleton and cell shape remained largely unaffected (Figure 10D, data not shown). These experiments indicate that a subset of NCAM shifts its localization to $p59^{Fyn}$/FAK containing lipid rafts upon the transition to a mesenchymal phenotype. Since NCAM has been shown to physically associate with FGFR, PLCγ and cortactin outside of lipid rafts (Cavallaro et al., 2001), we next assessed whether these NCAM-containing signaling complexes were unchanged during EMT.

Immunoprecipitation experiments with antibodies against NCAM revealed that FGFR2 was found associated with NCAM regardless of TGFβ treatment of NMuMG cells. In contrast, the signaling effectors PLCγ and cortactin co-immunoprecipitated with NCAM exclusively in untreated epithelial NMuMG cells and not in TGFβ-treated "mesenchymal" NMuMG cells (Figure 10E). These data suggest that during TGFβ-induced EMT of NMuMG cells NCAM changes its subcellular localization and its signaling partners, which in turn leads to focal adhesion stabilization.

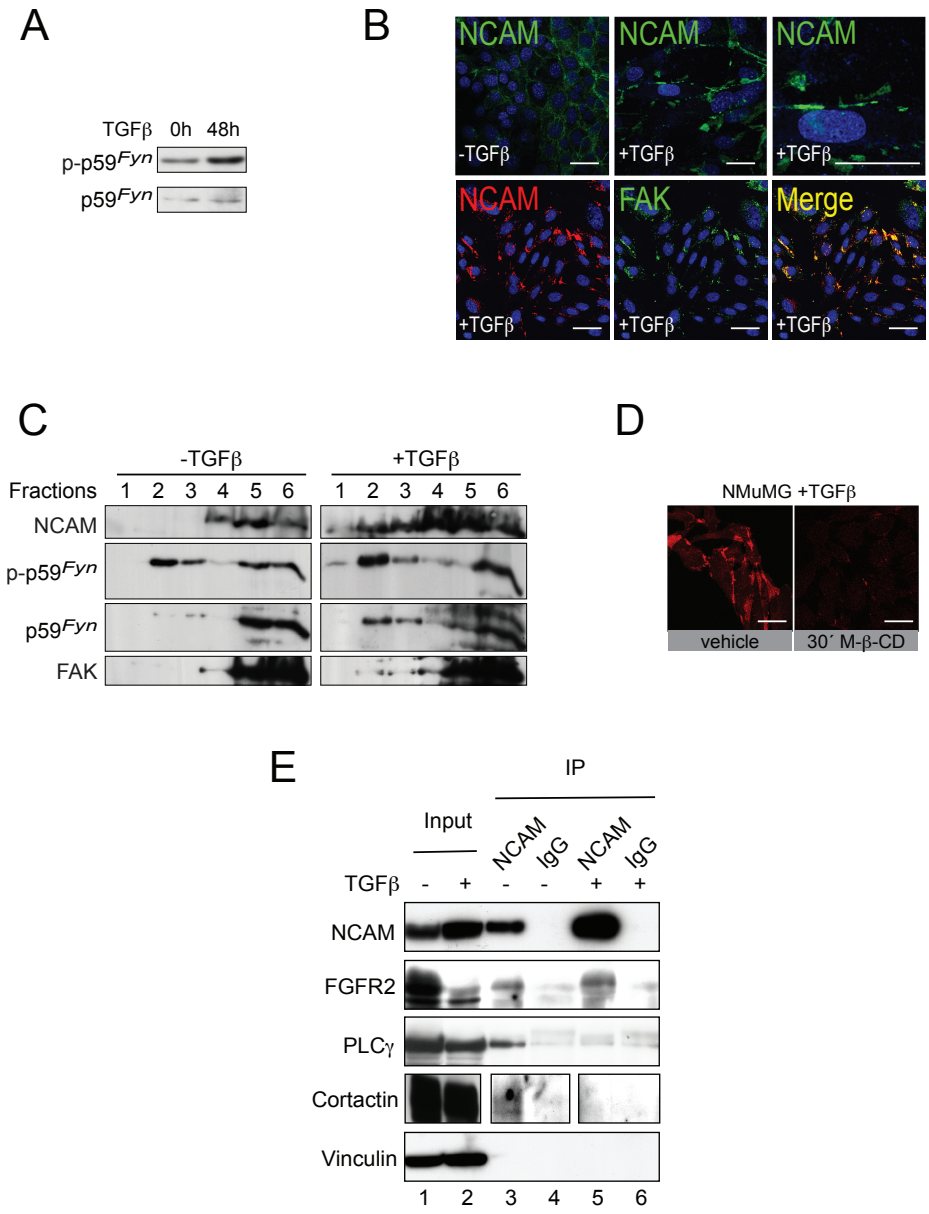


Figure 10. During EMT, NCAM localizes into lipid rafts together with p59^{Fyn}and FAK. **(A)** Immunoblotting analysis of NMuMG cells reveals an increase in p59^{Fyn} phosphorylation upon EMT induction (+TGFβ). Immunoblotting for total p59^{Fyn} was used as loading control. **(B)** Upper panels: Immunofluorescence staining for NCAM (green) in NMuMG cells in the presence or absence of TGFβ. During EMT, NCAM is translocated from sites of cell-cell contacts to foci at the plasma membrane (see higher magnification in top right panel). Lower panels: NCAM (red) and FAK (green) co-localize in TGFβ-treated NMuMG cells. DAPI staining (blue) visualizes nuclei. Size bars = 20 µm. **(C)** NCAM140 associates with lipid rafts upon induction of EMT. Lysates of TGFβ-treated or non-treated NMuMG cells were fractionated by sucrose gradient centrifugation and proteins were resolved by SDS-gel electorphoresis. After blotting, membranes were cut in upper and lower halves. The upper half was sequentially probed with antibodies against NCAM and FAK, the lower half was probed

with antibodies against phosphorylated-p59^{Fyn} and total p59^{Fyn}. In mesenchymal cells (+TGFβ), NCAM, p59^{Fyn} and FAK are present in lipid rafts (lanes 1 to 3) as well as in the Triton X-100 soluble high-density fractions (lanes 4 to 6). In epithelial cells (-TGFβ), they are only detected in the high-density fractions (lanes 4 to 6). **(D)** Immunofluorescence staining of NCAM (red) in NMuMG cells treated with TGFβ. Upon methyl-β-cyclodextrin (M-β-CD) treatment lipid rafts are disorganized and NCAM clusters disappear. Size bars = 20 μm. **(E)** NCAM forms a complex with FGFR2, PLCγ and cortactin in epithelial cells. Lysates from NMuMG cells treated or not with TGFβ were immunoprecipitated with anti-NCAM antibodies (NCAM) or unrelated isotype immunoglobulins (IgG). Total lysates and immunoprecipitates were resolved by SDS-gel electrophoresis, and after protein transfer the blot was sequentially probed with antibodies specific for PLCγ, NCAM, FGFR2 and vinculin. Cortactin was probed on a separate blot from a gel loaded with the same experimental cell extracts. Note that NCAM precipitates PLCγ and cortactin only in untreated NMuMG cells but not in TGFβ-treated cells.

3.1.3.7. NCAM mediates focal adhesion assembly in various EMT systems

To assess whether NCAM exerts this critical function also during EMT in other experimental systems, its expression was ablated in MCF7-shEcad, MTdeltaEcad and HEK293 cells by transfection of specific murine or human siRNAs (Supplementary Figure S6A-D). In MCF7-shEcad cells depleted from NCAM, a loss of neurite extensions was observed (Figure 11A), an established hallmark of NCAM function (Cavallaro et al., 2001). In contrast, no obvious morphological changes were observed in MTdeltaEcad cells upon NCAM depletion (data not shown), suggesting that these cells were irreversible in their mesenchymal phenotype (note that these cells carry a irreversible deletion of the *E-cadherin* gene).

As observed in TGFβ-treated NMuMG cells, numerous focal contacts were observed at the ventral surface of MTdeltaEcad and MCF7-shEcad cells (Figure 11B and data not shown). Consistent with this observation, increasing amounts of phosphorylated FAK and paxillin were observed in cells in MTdeltaEcad in comparison to their epithelial counterparts (Figure 11C). Upon depletion of NCAM from MTdeltaEcad cells, FAK phosphorylation was rapidly lost during cell spreading, while it remained constant in control cells (Figure 11D). As a consequence, cell spreading of NCAM-depleted MCF7-shEcad, MtdeltaEcad and HEK293 cells were reduced (Figure 11E and data not show). Notably, phosphorylation of paxillin was unaffected by the presence or absence of NCAM, indicating that paxillin phosphorylation is mediated by an NCAM-independent pathway (Figures 11D, 9C,D).

Comparable to NMuMG cells, in HEK293 cells NCAM localization also shifted from non-lipid raft to lipid raft membrane fractions upon loss of E-cadherin (Figure 11F). Consistent with this observation, disruption of lipid rafts by cholesterol depletion with M-β-

CD completely impaired the formation of NCAM clusters in HEK293-shEcad and MTdeltaEcad cells (Figure 11F and data not shown). Forced, inducible expression of NCAM in HEK293 cells resulted in the translocation of a subset of NCAM into lipid rafts, demonstrating that increased levels of NCAM protein mediate its translocation to lipid rafts and that the loss of E-cadherin is not necessarily required for this process (Supplementary Figure S7).

Immunoprecipitation experiments revealed that in E-cadherin-deficient HEK293-shEcad cells the high levels of NCAM associated with $p59^{Fyn}$, an interaction that could not be detected in control cells expressing low levels of NCAM (Figure 11G). Similarly to TGFβ-treated NMuMG cells, in HEK293 cells NCAM associated with PLCγ only in the presence of E-cadherin (Figure 11G). These results indicate that, upon loss of E-cadherin function, NCAM ceases to associate with PLCγ and cortactin and a subset of NCAM protein re-localizes to lipid rafts where it binds to $p59^{Fyn}$.

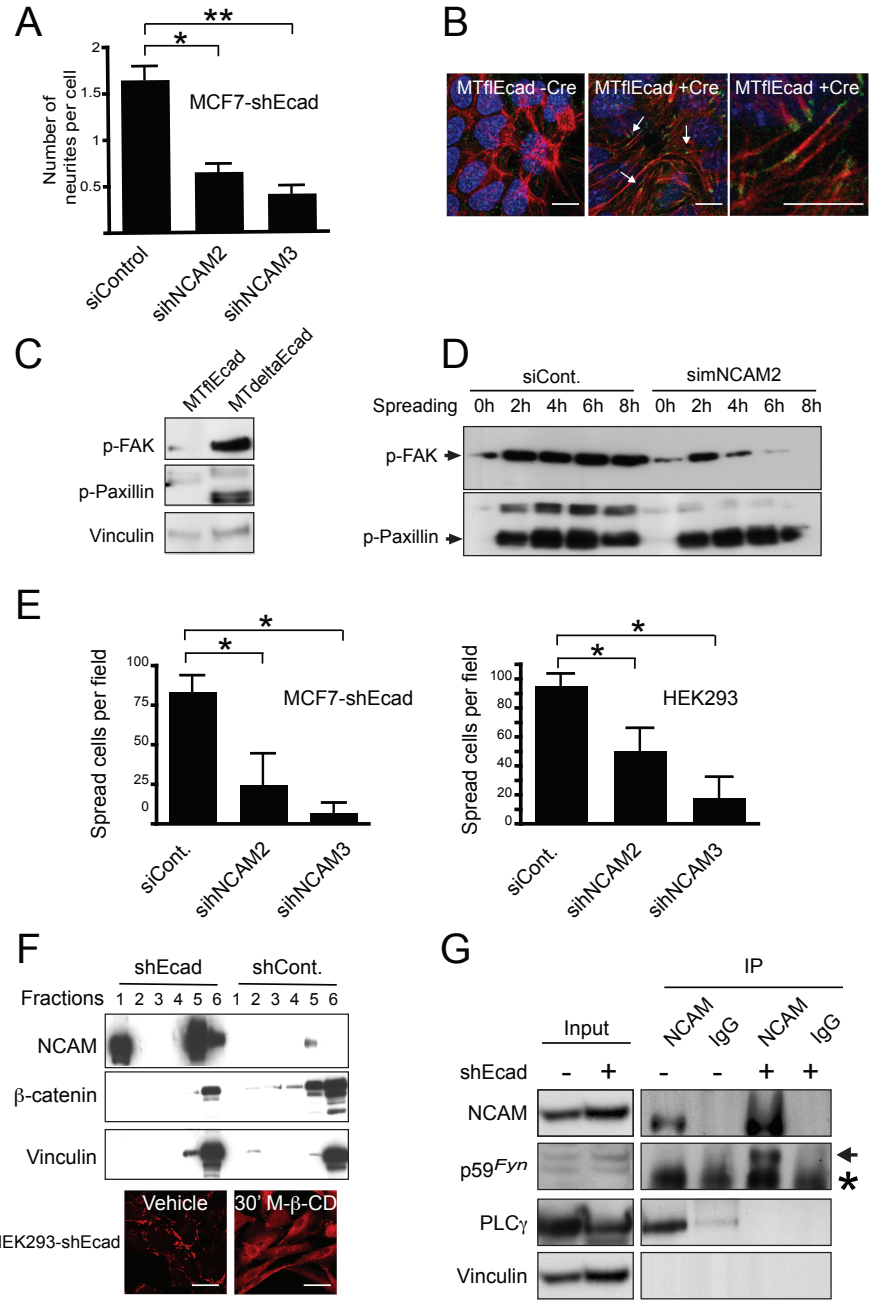


Figure 11. NCAM expression control focal adhesion assembly in various mesenchymal cells. **(A)** Quantification of neurites in MCF7-shECad cells reveals that the number of neurites per cell is significantly decreased in siNCAM-transfected cells compared to siControl-transfected cells (lower panel, *p=0.0133 and **p=0.0087, unpaired t-test). Data are shown as mean±SD. **(B)** Immunofluorescence staining for actin (red) and $p125^{FAK}$ (green) on MTflEcad cells with and without Cre-recombinase expression. Numerous focal adhesions are detected in E-cadherin-depleted cells (arrows, middle panel) as compared to control cells (left panel). Higher magnifications of the middle panels are shown on the right. Staining with

DAPI (blue) visualizes nuclei. Size bars = 10 µm. **(C)** Immunoblotting analysis of MTflEcad and MTdeltaEcad cells reveals an increase in FAK and paxillin phosphorylation upon mesenchymal transition. Immunoblotting for vinculin was used as loading control. **(D)** MTdeltaEcad cells were transfected with siControl or siNCAM SiRNA, allowed to spread for the indicated times and the levels of phosphorylation of FAK and paxillin were determined by immunoblotting analysis. Cell lysates were resolved by SDS gel electrophoresis, and after protein transfer blots were sequentially probed with antibodies against phosphoryalated FAK and phosphorylated paxillin. **(E)** Cell spreading of MCF7-shEcad and HEK293 cells transfected with different siRNAs targeting NCAM (sihNCAM2 and 3) on plastic culture dishes is reduced as compared to control-treated cells (siCont). (*p=0.0021, unpaired t-test). Data are shown as mean±SD. **(F)** NCAM140 associates with lipid rafts upon loss of E-cadherin expression. Upper panels: Lysates of HEK293-shECad and HEK293-shCont cells were fractionated by sucrose gradient centrifugation and analyzed by immunoblotting for NCAM, β-catenin and vinculin as loading control. In E-cadherin-depleted cells (shEcad), NCAM is present in lipid rafts (fractions 1 and 2) as well as in the Triton X-100 soluble high-density fractions (fractons 5 and 6). In the presence of E-cadherin (shCont), NCAM is only detected in the high-density fractions (fractions 5 and 6). Lower panels: Immunofluorescence staining of NCAM (red) in HEK293-shECad cells treated with methyl-β-cyclodextrin (M-β-CD). Upon treatment, lipid rafts are disorganized and NCAM clusters disappear. Size bars = 20 µm. **(G)** HEK293-shEcad (shEcad +) and HEK293-shCont (shEcad -) cell lysates were immunoprecipitated with antibodies against NCAM or an unrelated immunoglobulin isotype (IgG). NCAM precipitated $p59^{Fyn}$ (arrow) only in the absence of E-cadherin, and PLCγ only in the presence of E-cadherin. Vinculin was used as loading control. The asterisk indicates immunoglobulin heavy chain.

3.1.3.8. Expression of NCAM in human and mouse tumors

We next determined whether up-regulated NCAM expression was also observed in E-cadherin-negative cancer cells *in vivo*. NCAM and E-cadherin expression were analyzed by immunohistochemical staining of tumor tissue from a recently published mouse model of lobular breast carcinogenesis, in which mice carrying LoxP-flanked alleles of the p53 gene (Trp53F) were crossed with keratin14 gene-promoter-Cre recombinase mice (K14-Cre) and with mice carrying LoxP-flanked alleles of E-cadherin (EcadF/F; (Derksen et al., 2006). In E-cadherin-expressing tumors (K14-Cre;Trp53F/F;EcadF/+), E-cadherin was expressed at the membrane of most tumor cells (Figure 12A), while NCAM could only be detected in large blood vessels of the surrounding fat or muscle tissue and in the basal layer of the epidermis (Figure 12A,E). In contrast, in tumors of K14-Cre;Trp53F/F;EcadF/F mice, E-cadherin expression was efficiently ablated (Figure 12A,C), yet NCAM expression was now detectable at varying intensities in tumor cells (Figure 12A,D). In particular, cells within invasive tumor fronts displayed significant NCAM expression, for example at the interface between tumors and subcutaneous myocytes and adipocytes, as well as in disseminated tumor cells (Figure 12A,E and F).

We next assessed NCAM expression in human cancers by immunohistochemical staining of

multi-tissue arrays containing 39 different human cancer types and corresponding 22 normal tissues. Depending on availability, between 5 and 50 samples per cancer type were analyzed. A particular strong up-regulation of NCAM was observed in neuroendocrine tumors, including small-cell lung cancer (SCLC, 35 of 43 patients, $p < 0.001$, Fisher's exact test) and intestinal carcinoids (33 of 44 patients, $p < 0.001$, Fisher's exact test), cancer types known to lose E-cadherin expression during tumor progression (Salon et al., 2004). Staining of serial histological sections of a human intestinal neuroendocrine carcinoid revealed expression of NCAM predominantly in the invasive cancer cells of the tumor with low or no E-cadherin expression, while NCAM was not found to be expressed in the E-cadherin-positive, differentiated compartments of the tumor (Figure 12B). These results indicate that NCAM is upregulated upon loss of E-cadherin function in certain types of human cancers.

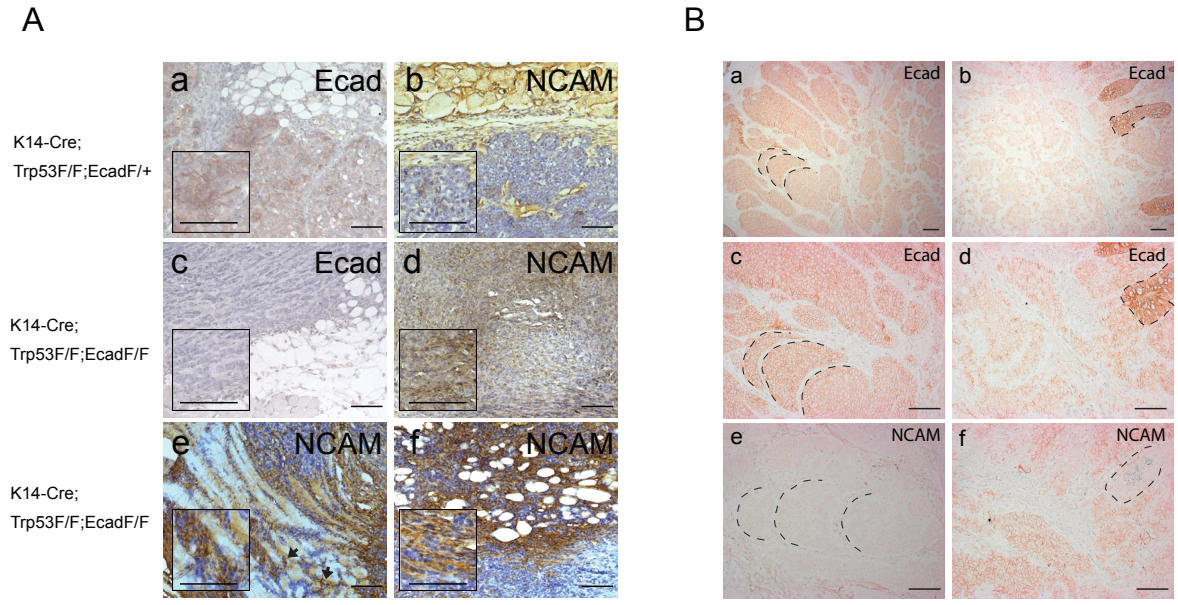


Figure 12. NCAM is up-regulated in E-cadherin-negative tumors. **(A)** Serial histological sections from breast tumors of

K14-Cre;Trp53F/F mice expressing E-cadherin (Ecad F/+) or deficient in E-cadherin expression (Ecad F/F) were stained with antibodies against E-cadherin **(a** and **c)** and NCAM **(b**, **d**, **e** and **f)**. NCAM is absent in E-cadherin-expressing tumor cells (**a**, **b**). In contrast, NCAM is expressed in tumors that have lost E-cadherin expression (**c**, **d**). NCAM is predominantly expressed on the invasive front of E-cadherin-negative tumors at the interphase between tumor cells and myocytes (**e**) or adipocytes of the skin (**f**). Disseminated tumor cells in the stroma (indicated by arrows in **e**) also display strong NCAM staining. Inserts represents higher magnifications of the respective sections. Size bars = 100 µm. **(B)** Serial histological sections from a human patient neuroendocrine carcinoid were stained with antibodies against E-cadherin **(a-d)** and NCAM **(e,f)**. NCAM is not expressed in E-cadherin-positive, differentiated cancer cells, whereas NCAM is expressed in invasive cancer cells with reduced E-cadherin expression. Morphological structures are marked for orientation. Size bars = 200 µm.

3.1.4. Discussion

We have employed cellular experimental systems, transgenic mouse models and patient biopsies to investigate how the loss of E-cadherin function contributes to tumor progression. Notably, E-cadherin depletion in cultured cells *in vitro* leads to increased expression of the cell adhesion molecule NCAM. A comparable NCAM up-regulation is also observed in transgenic mouse models of malignant insulinoma and lobular breast carcinoma and in human small-cell lung cancer and intestinal carcinoids, where a loss of E-cadherin and a gain of mesenchymal markers have been previously implicated in tumor progression (Salon et al., 2004). Interestingly, expression of L1, a close relative of NCAM, is also observed in E-cadherin-negative cells of the invading tumor front, for example in colorectal cancer (Gavert et al., 2005).

The molecular mechanisms underlying the regulation of *NCAM* gene expression during EMT are the focus of intense investigations in our laboratory. Here, we have assessed the functional contribution of upregulated NCAM expression to EMT. In the tumor progression models employed here, NCAM is found in two distinct complexes: in the presence of E-cadherin low levels of NCAM localize outside of lipid rafts and associate with FGFR, PLCγ and cortactin. Upon loss of E-cadherin function, NCAM expression levels are increased, and a subset of NCAM protein translocates to lipid rafts, where it associates with $p59^{Fyn}$. Subsequent phosphorylation and activation of $p59^{Fyn}$ and its effector substrate FAK result in the stabilization of β_1 integrin-mediated focal adhesions and increased cell spreading and migration (Figure 13). On the other hand, FGFR transmits NCAM-dependent and NCAM-independent signals: NCAM-mediated activation of FGFR leads to the activation of PLCγ and via the Raf-kinase PKCβII to sustained MAPK activation and increased cell

adhesion. In contrast, stimulation of FGFR by FGFs activates PLCγ/PKCα and Ras/Raf/MAPK signaling pathways and promotes cell adhesion and proliferation (Francavilla et al., 2007); Figure 13). Thus, loss of E-cadherin affects the nature of NCAM-mediated signal transduction by inducing *NCAM* gene expression and by changing NCAM's subcellular localization. Forced expression of NCAM also resulted in its translocation to lipid rafts even in the presence of E-cadherin, indicating that the levels of NCAM proteins determine its subcellular localization and thus its signaling qualities (Supplementary Figure S7). It is interesting to note that *NCAM* is a haploinsufficient gene, indicating that *NCAM* gene dosage plays a critical role in development and disease (Esni et al., 1999; Perl et al., 1999). Our results may provide a biochemical explanation for this gene dosage effect. The results also substantiate findings in neurons, where it has been demonstrated that NCAM140 localized in membrane compartments outside of lipid rafts binds and stimulates FGFR via its fibronectin type III domains, which in turn activates PLCγ and MAPK signaling pathways (Doherty and Walsh, 1996; Niethammer et al., 2002). In contrast, when situated within lipid rafts, NCAM associates with $p59^{Fyn}$ and activates FAK kinase-mediated signaling (Beggs et al., 1997; Niethammer et al., 2002).

Apparently, NCAM expression plays an essential role in mesenchymal cell motility as characterized by the formation of β_1-integrin-dependent focal adhesions. We have previously shown that the genetic ablation of *NCAM* gene expression results in the loss of β_1-integrin activation (Cavallaro et al., 2001) and that ablation of NCAM or β_1-integrin leads to reduced tumorigenicity of tumor cells (Kren et al., 2007). Here we demonstrate that, after loss of E-cadherin function and gain of NCAM expression during EMT, phosphorylated FAK is stabilized and β_1-integrin-mediated cell spreading is enhanced. In contrast, in epithelial cells, FAK phosphorylation has a high turnover, very few focal adhesions are detected, and cell spreading is slow and largely β_1-integrin-independent.

Based on the data presented, we conclude that the loss of E-cadherin, among many other molecular consequences, induces up-regulated expression of the *NCAM* gene. As a result, increased levels of NCAM localize to lipid rafts and via $p59^{Fyn}$ induce the formation of focal adhesions and integrin-dependent mesenchymal cell migration and invasion. The correlation

between the loss of E-cadherin and the gain of expression of NCAM and other Ig-domain adhesion molecules in specific human cancer types and mouse models of carcinogenesis indicate that these molecular pathways play a critical role in malignant tumor progression and metastasis and, thus, warrant further investigation.

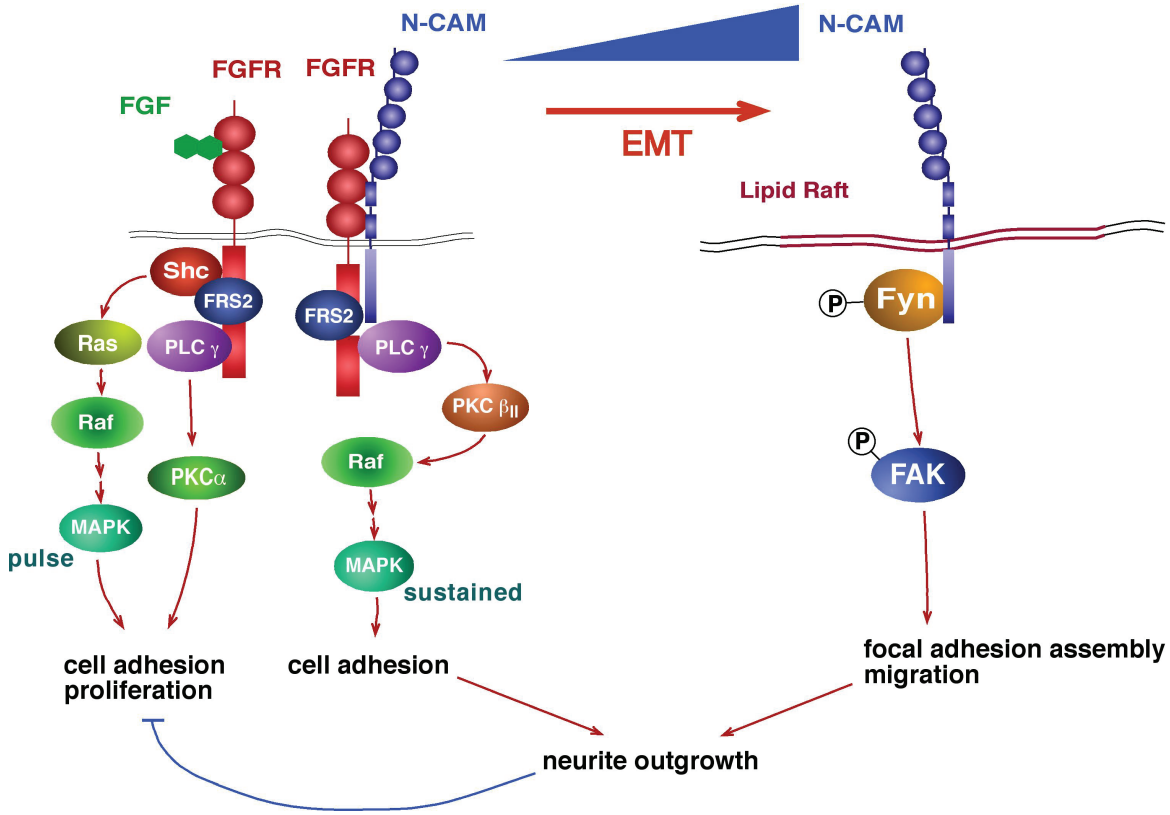


Figure 13. NCAM upregulation leads to a functional switch. Depicted is a working model of the molecular mechanisms underlying the change in NCAM subcellular localization and its interacting partners during EMT. In epithelial cells, low levels of NCAM form a complex with FGFR and PLCγ leading to the activation of the Raf-kinase PKCβII and thus to a sustained activation of the MAPK pathway and cell adhesion. Upon loss of E-cadherin function during EMT, NCAM is highly expressed, and a subset localizes to lipid rafts where it associates with $p59^{Fyn}$, leading to FAK phosphorylation, focal adhesion assembly and cell migration. Both NCAM-mediated signaling pathways are required for neurite outgrowth. In contrast, FGF-induced stimulation of FGFR results in PLCγ-mediated activation of PKCα and a short pulse activation of the Ras/Raf/MAPK pathway resulting in cell adhesion and proliferation. FGF-induced signaling is overruled by the NCAM-

mediated signals (Cavallaro et al., 2001; Francavilla et al., 2007).

3.1.5. Supplementary Figures

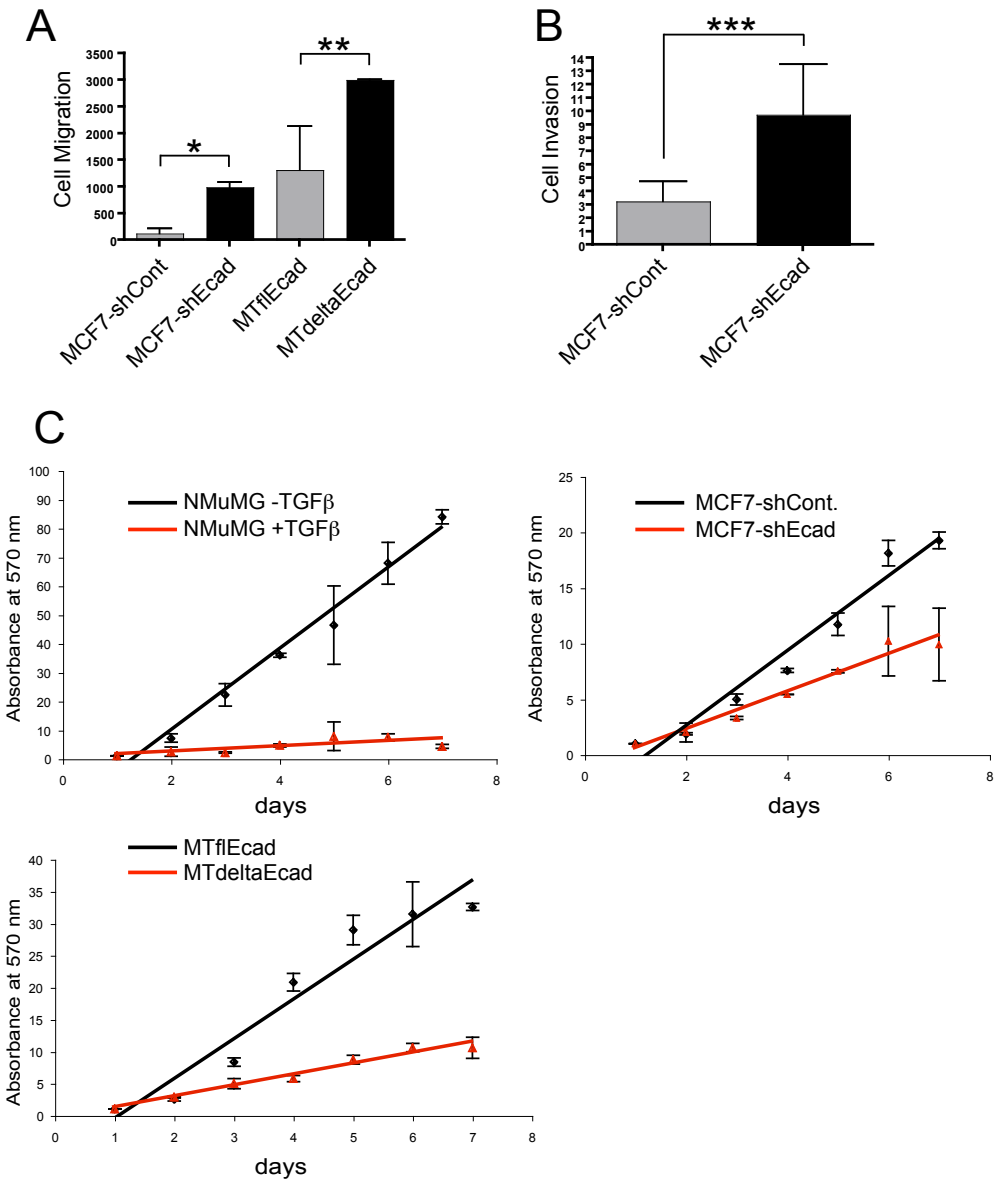


Figure S1. Increased migration of MCF7 and MTflEcad cells upon loss of E-cadherin (shEcad and MTdeltaEcad, respectively). *p<0.0001 and **p= 0.0236, unpaired t-test. Data are shown as mean±SD. (B) shRNA-mediated depletion of E-cadherin (shEcad) induces invasion of MCF7 cells. ***p=0.0186, two sided t-test. Data are shown as mean±SD. (C) Epithelial-mesenchymal transition reduces the proliferation rates of NMuMG, MCF7 and MTflEcad NMuMG cells. Cell numbers were measured using an MTT assay.

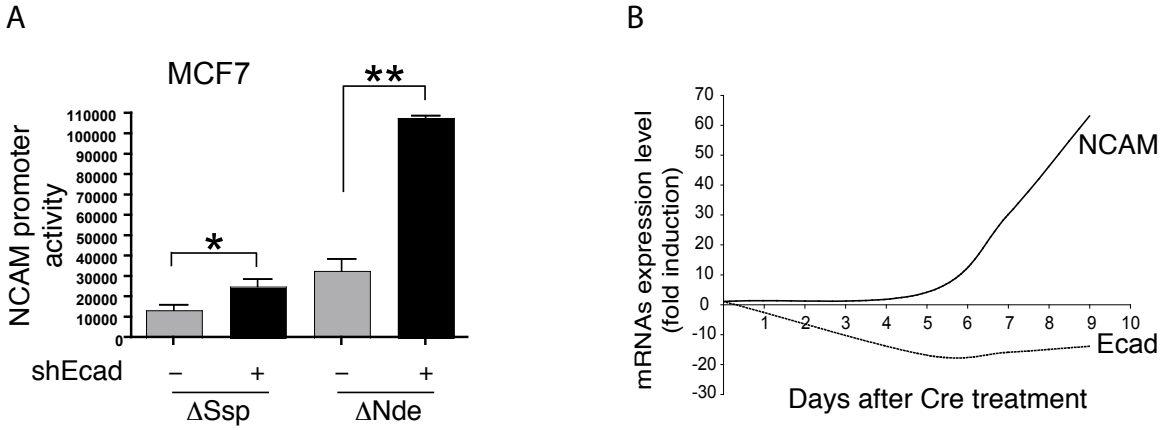


Figure S2. NCAM promoter activity is increased upon loss of E-cadherin. (A) MCF7 cells expressing E-cadherin or depleted for E-cadherin expression were transfected with a CAT reporter plasmid containing 1000 bp (DSsp) or 647 bp (DNde) of the NCAM promoter sequence, and CAT activity was determined 48 hours after transfection (*p = 0.0114 and **p = 0.0374). Data are shown as mean±SD. (B) MTflEcad cells carrying floxed alleles of the E-cadherin gene were treated with purified recombinant Cre-recombinase fused to a HIV-Tat entry peptide to ablate the E-cadherin alleles by recombination/excision. RNA was extracted at the time points of treatment as indicated, and the relative expression levels of NCAM and E-cadherin were determined by quantitative RT-PCR.

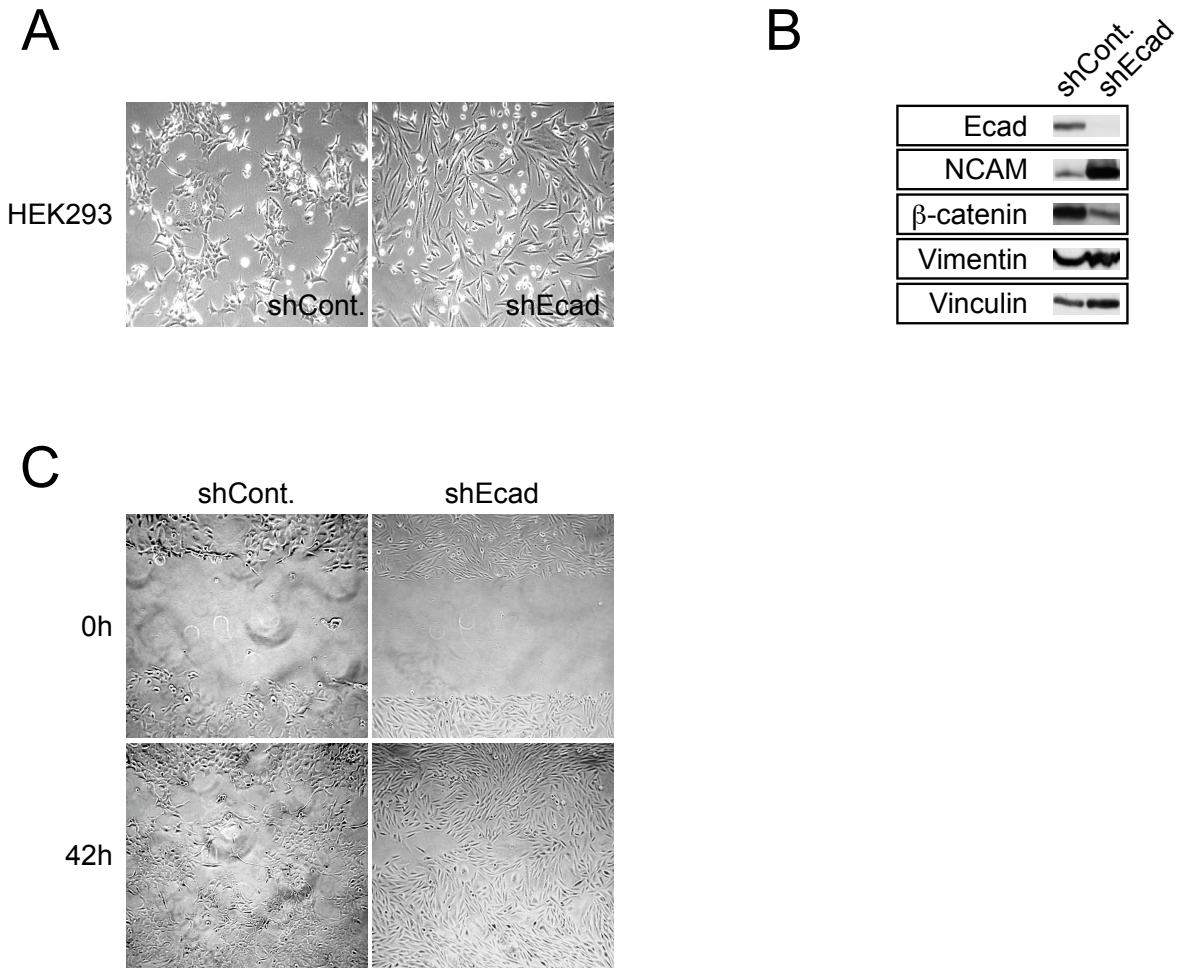


Figure S3. Loss of E-cadherin expression in HEK293-shEcad cells. (A) Phase contrast micrographs of HEK293 cells stably expressing a control shRNA (shCont, left panel) or an E-cadherin-specific shRNA (right panel). (B) Immunoblotting analysis of E-cadherin (Ecad), NCAM, b-catenin, and vimentin expression in HEK293-shCont and HEK293-shEcad cells. Immunoblotting for vinculin was used as loading control. (C) Scratch wounding assay on confluent layers of HEK293-shCont and HEK293-shEcad cells. 42 hours after wounding, phase contrast microphotographs were taken. Note that within this period, HEK293-shEcad cells had almost closed the gap, while HEK293-shCont cells had migrated less.

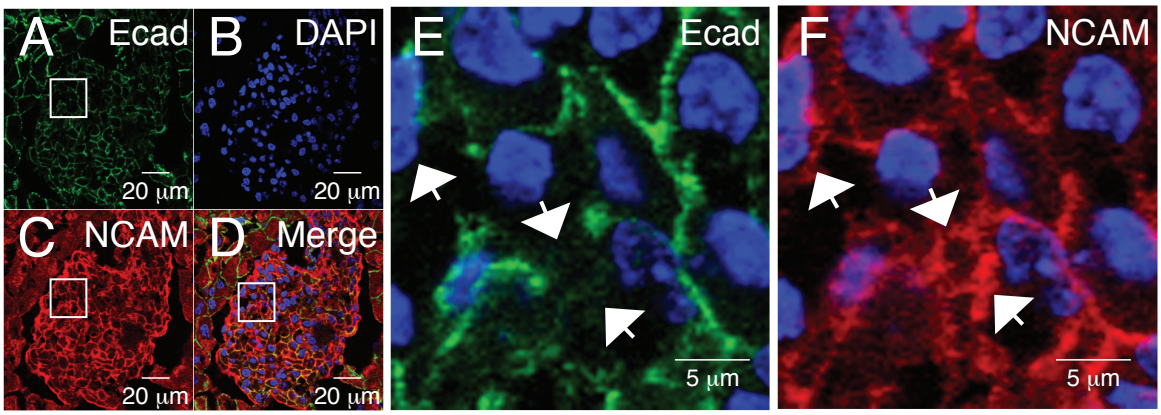


Figure S4. E-cadherin deletion in b cells of the islets of Langerhans leads to increased NCAMexpression in normal tissues. Immunofluorescence staining for E-cadherin (green; A, D, E) and for NCAM (red; C, D, F) in a pancreatic section from a E-cad F/F;RipCre mouse as indicated. E and F represent higher magnifications of the boxes in panels A and C, respectively. Single b cells that have lost E-cadherin expression and gained high levels of NCAM expression are indicated by arrows. Nuclei are stained with DAPI (blue).

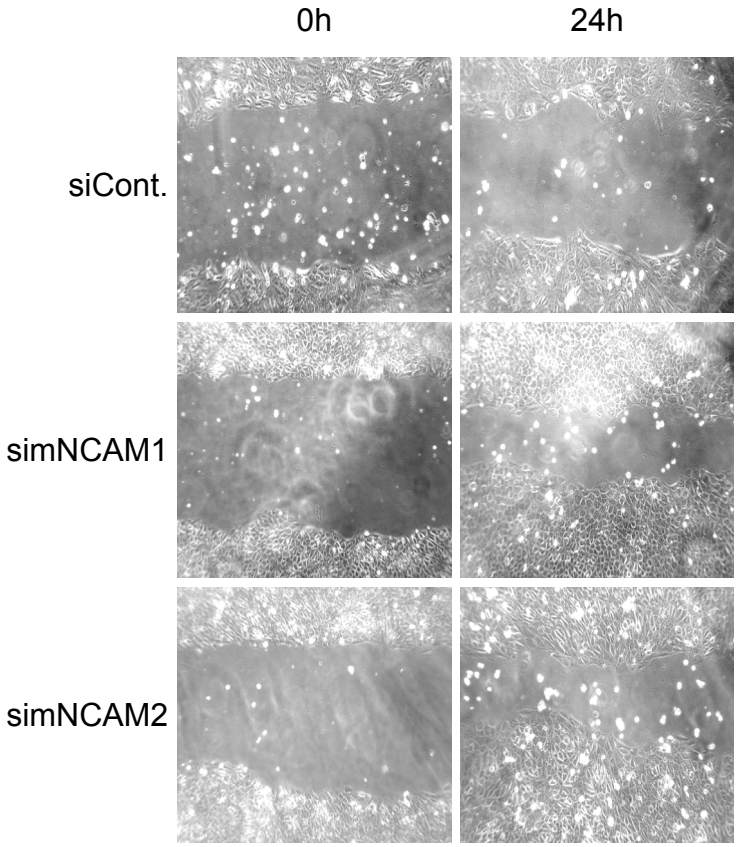


Figure S5. NCAM depletion increases cell migration Scratch wounding assay on confluent layers of NMuMG cells transfected with siControl or siNCAM siRNA and treated with TGFβ. 24 hours after wounding, phase contrast microphotographs were taken. Note that within this time period, siNCAM-transfected cells had almost closed the gap, while the siControl cells had migrated less.

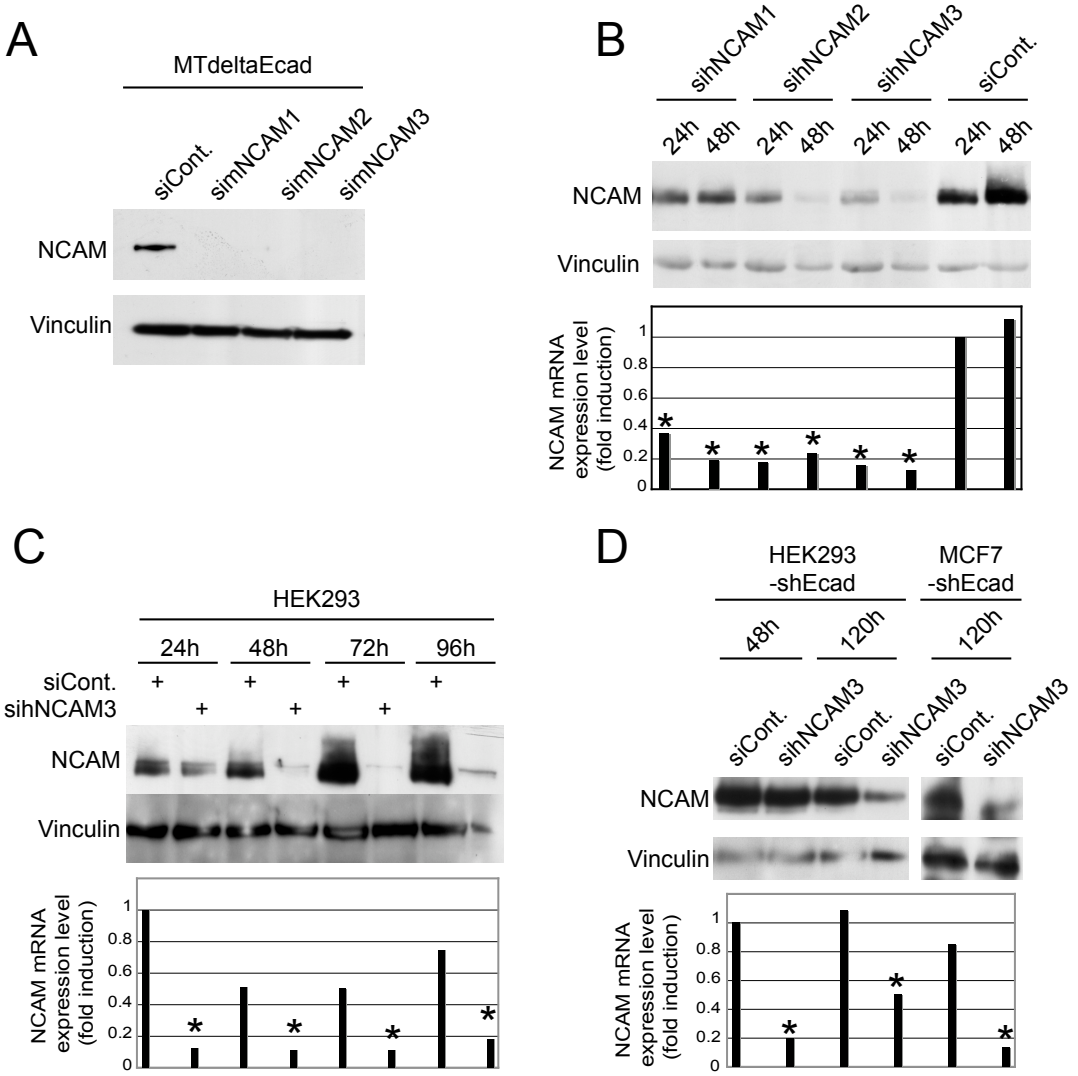


Figure S6. Characterization of siRNA targeting mouse and human NCAM. (A) Immunoblotting analysis of MTdeltaEcad cells transfected for 48 hours with mouse NCAM-specific siRNA targeting NCAM or with mismatch control siRNA. Immunoblotting for vinculin was used as loading control. (B and C) HEK293 and HEK293-shEcad and MCF7-shEcad cells (D) were transfected for the indicated times with 3 independant siRNA targeting human NCAM or with mismatch control siRNA (siCont) and analyzed by immunoblotting (upper panels) or quantitative RT-PCR (lower panels) for NCAM expression. Mouse riboprotein L19 primers were used for normalization, and fold induction was calculated against siControl-treated cells using the comparative Ct method (ΔΔCt). *p<0.01, unpairedt-test. Data are shown as mean±SD.

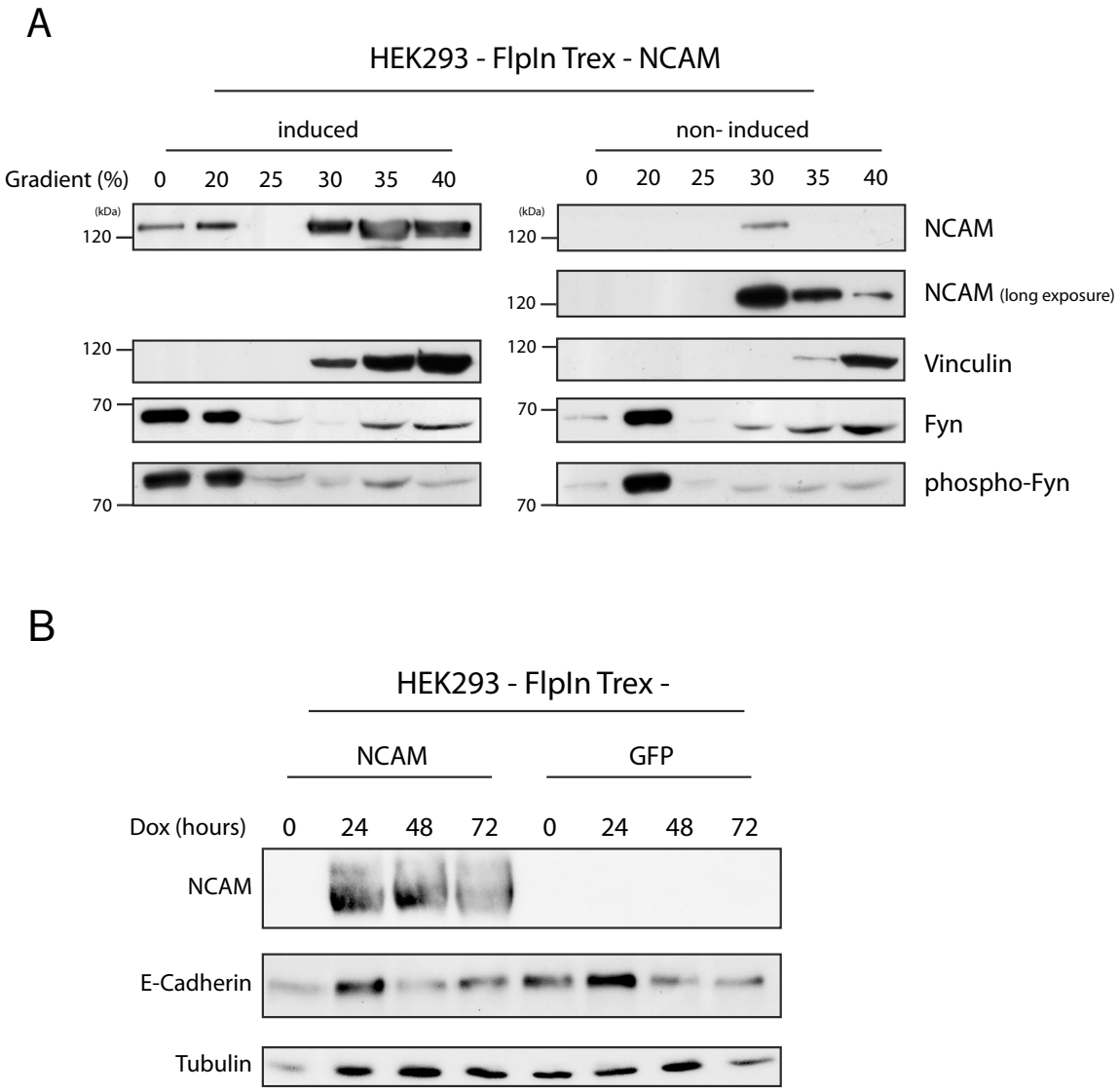


Figure S7. High levels of NCAM140 associate with lipid rafts. (A) HEK293 cells expressing NCAM under the control of the Tetracycline-inducible system (HEK293-FlpInTrex-NCAM) were either grown in the absence of Doxycycline (non-induced) or in the presence of Doxycycline (induced) to induce expression of NCAM. Lysates of the cells were fractionated by sucrose gradient centrifugation and analyzed by immunoblotting for NCAM, phosphorylated-p59Fyn, p59Fyn and vinculin, as indicated. In cells expressing high levels of NCAM (induced), NCAM, p59Fyn and FAK are present in lipid rafts (0 and 20% sucrose) as well as in the Triton X-100 soluble high-density fractions (30, 35, and 40% sucrose). In cells expressing low levels of NCAM, they are only detected in the high-density fractions (30, 35, and 40% sucrose).(B) Expression levels of E-cadherin remain unchanged upon induced expression of NCAM. HEK293-FlpInTrex-NCAM and HEK293-FlpInTrex-GFP cells were induced with doxycycline to express NCAM and GFP, respectively, and NCAM and E-cadherin levels were determined by immunoblotting at the time points indicated. Tubulin served as loading control.

3.1.6. Material and Methods

Reagents and antibodies

Mouse NCAM-Fc (20 μg/ml) was a gift from U. Cavallaro (IFOM, Milano, Italy). TGFβ: R&D Systems (Abingdon, UK). Antibodies: anti-α-actin (Abcam, Cambridge, UK), anti-E-cadherin (for immunoblotting), anti-pY397FAK, anti-FAK, anti-p-Paxillin, anti-pY528p59^{Fyn} (BD Transduction Laboratories, Allschwil, Switzerland), anti-E-cadherin (for immunostaining; Zymed, San Diego, CA), anti-vinculin N-19, and anti-hNCAM 123C3 (Santa Cruz Biotechnology, Heidelberg, Germany), anti-vimentin clone V9, anti-β-catenin and anti-hNCAM OB-11 (Sigma, Basel, Switzerland), anti-β_1-integrin MAB2253Z (Millipore, Volketswil, Switzerland), anti-FGFR2 C-17, anti-cortactin 4F11, anti-PLCγ and anti-p59^{Fyn} (Upstate Biotechnology, Lake Placid, NY), anti-murine NCAM 5B8 (immunoblotting and immunofluorescence), anti-NCAM 13 (immunoprecipitation; BD Transduction Laboratories) and anti-N-cadherin (Takara Biomedicals, Tokyo, Japan).

Cells and cell lines

A subclone of NMuMG cells (NMuMG/E9; hereafter NMuMG) expressing E-cadherin has been previously described (Maeda et al., 2005). MDCKII, NMuMG, HEK293 and MCF7 cells were cultured in DMEM supplemented with glutamine, penicillin, streptomycin, and 10% FCS (Sigma). Oligonucleotides (Suppl. data, Table S1) were annealed and inserted into the pSUPER-retro-neo vector (OligoEngine, Seattle, WA) to generate pSUPER-Ecad-shRNA and pSUPER-Cont-shRNA. MCF7 and HEK293 cells stably transfected with pSuperRetro-Ecad-shRNA (shEcad) and pSuperRetro-mNCAM-shRNA (sh-cont) and MDCKII cells stably transfected with NCAM140 expression vectors were selected in G418. NMuMG-shSmad4 and NMuMG-shCont were obtained from P. ten Dijke (Leiden University Medical Center, The Netherlands; (Deckers et al., 2006). TGFβ treatment of NMuMG cells was performed without serum deprivation, and TGFβ was replenished every 3 days. MTflEcad cells were established from a mammary gland tumor isolated from an MMTV-Neu mouse

(Muller et al., 1988) crossed with EcadF/F mice (Derksen et al., 2006). To establish the MTflEcad-Cre cell line, MTflEcad cells were infected with LZRSpBMN retroviral vector expressing Cre-IRES-GFP. After infection, GFP-expressing cells were sorted by FACS. hNCAM and mNCAM siRNAs were purchased from Invitrogen (Stealth siRNA duplex oligoribonucleotides; Table S1). Transfections with LipofectAMINE RNAiMAX (Invitrogen) were performed according to the manufacturer's instructions. Total cell lysates, immunoblots, and immunofluorescence experiments were performed as previously described (Wicki et al., 2006). Depending on the species origin of antibodies, immunoblots were either probed sequentially or on multiple membranes. Adobe Photoshop has been used to excise the relevant portion of the immunoblots from the original scans of X-ray films exposed to chemiluminescence visualization of specific proteins, as indicated by black frames in the figures.

Migration and invasion assays

Cell migration was determined in a modified two-chamber migration assay (pore size: 8 μm; Falcon BD, Franklin Lakes, NJ). For invasion assays, membranes were coated with 20 μl of a 2.5 mg/ml solution of Matrigel (Falcon BD). For both assays, 10^5 cells were seeded in 1% fetal calf serum/DMEM (Sigma) in the upper chamber and the lower chamber was filled with 10% fetal calf serum/DMEM. After 24h incubation at 37°C, cells in the upper chamber were carefully removed with a cotton swap and the cells that had traversed the membrane were fixed in 4% paraformaldehyde/HBS-Ca^{2+}, stained with crystal violet (0.5% in 20% methanol) and counted.

Cell spreading assay

To quantify cell spreading, cells were plated on a plastic culture dish, and 30 minutes after seeding the medium was changed to eliminate unattached cells. Cells were incubated at 37°C and counted in regular intervals in four independent experiments. In cases where β_1-integrin blocking antibodies were used, cells were incubated for 30 minutes with 100 μg/ml mouse IgG or anti-β_1-integrin antibodies prior to seeding.

In vitro wounding assay

A scratch wound was generated using a 200 µl pipette tip on confluent cell monolayers in a 6-well culture plates in 10% serum containing DMEM-medium (Sigma, St. Louis, Missouri). Cells were then washed with fresh medium to remove floating cells. Microphotographs were taken at different time points.

Quantitative RT-PCR

Total RNA was prepared using Trizol (Invitrogen), reverse transcribed with M-MLV reverse transcriptase RNAse (H-) (Promega, Wallisellen, Switzerland), and transcripts were quantified by PCR using SYBR-green PCR MasterMix (Applied Biosystems, Rotkreuz, Switzerland) and the primers indicated in Table S1. Human or mouse riboprotein L19 primers were used for normalization. PCR assays were performed in triplicates, and fold induction was calculated against control-treated cell lines using the comparative Ct method ($\Delta\Delta$ C_t).

CAT assays

NMuMG cells were transfected with ΔNde and ΔSsp NCAM-CAT reporter constructs(Boras and Hamel, 2002) and treated with TGFβ for 6 days. CAT assays were performed using the CAT Enzyme Assay System (Promega). CAT activities were normalized relative to co-transfected control luciferase activities.

Immunoprecipitations

Cells were lysed in RIPA-plus buffer. Equal amounts of proteins were incubated overnight with either mouse IgG or with anti-NCAM antibodies. Protein G sepharose beads (Sigma) were incubated with the immune complexes for 30 minutes, washed in cold lysis buffer and boiled in 2x SDS-PAGE loading buffer.

Flotation assays

Cells were lysed in cold lysis buffer for 30 minutes (1% Triton X-100, 25 mM Tris-HCl pH

7.5, 10% sucrose, 1 mM $CaCl_2$, 1 mM $MgCl_2$ containing 1 mM NaF, 2 mM Na_3VO_4, 0.1 mM PMSF, 1 mM DTT and protease inhibitor cocktail), and isolation of Triton X-100-insoluble membrane fractions by sucrose gradient density centrifugation was performed as described (Oliferenko et al., 1999).

Histological analysis

Mice with a deletion of both E-cadherin alleles specifically in the β cells of the islets of Langerhans were generated by crossing mice carrying E-cadherin alleles flankes by loxP sites (E-cadfl/fl; (Derksen et al., 2006) with RipCre mice (Ahlgren et al., 1998). Mice were sacrificed at the age of 56 days. The preparation of frozen tissue sections and immunofluorescence and immunohistochemical analysis was performed as described previously (Perl et al., 1998); (Wicki et al., 2006). In case of mouse-anti-mouse antibodies, background was reduced by additional blocking with the m.o.m kit (Vector Laboratories, Burlingame, California) according to the manufacturer's recommendations. The following primary antibodies were used at a 1:100 dilution: rat anti-mouse E-cadherin (Zymed), mouse anti-human E-cadherin (BD), anti-mouse NCAM 5B8 (a gift from U. Cavallaro), and mouse anti-human NCA (OB11, Sigma). Stainings were evaluated on an AxioVert microscope and on a LSM 510 META confocal microscope (Zeiss, Oberkochen, Germany). Human cancer samples were obtained with the consent of the Ethical Committee of the Kantons beider Basel.

Statistical analysis

Statistical analysis and graphs were generated using the GraphPad Prism software (GraphPad Software Inc, San Diego, CA). All statistical analysis were done by unpaired, two-sided t-test. Normality testing was performed using the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie for p-values.

3.2. Dlx2 protects NMuMG cells from TGFβ induced cell-cycle arrest and apoptosis

Mahmut Yilmaz and Gerhard Christofori. Manuscript in preparation.

3.2.1. Abstract

Resistance against TGFβ-induced growth inhibition is a pre-requisite for tumor progression and metastasis formation. Tumor cells evade TGFβ-mediated growth inhibition by attenuating canonical TGFβ signaling and by undergoing an epithelial-mesenchymal transition (EMT).

Here, we demonstrate that the transcription factor distal-less homeobox 2 (Dlx2) is upregulated during EMT by canonical TGFβ signaling. Dlx2 induces the transcriptional repression of the transforming growth factor receptor II (TGFβRII), leading to attenuated canonical TGFβ signaling and thus reduced expression of TGFβ target genes, such as cyclin-dependent kinase inhibitor $p21^{CIP1}$. Simultaneously, Dlx2 promotes survival and proliferation via epidermal growth factor receptor (EGFR)-mediated activation of the mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) signaling pathways. Ablation of Dlx2 expression during EMT decreases survival and impairs the ability of B16 melanoma cells to grow into primary tumors in transplanted syngeneic mice and to metastasize to the lung. Conversely, increased expression of Dlx2 during EMT promotes survival and correlates with the invasiveness and metastatic potential of human cancers.

These results establish a mechanistic link between Dlx2 expression, resistance against TGFβ-mediated growth inhibition and promotion of cell survival and invasion.

3.2.2. Introduction

The chemokine TGFβ plays a central role in various biological processes such as development, tissue homeostasis and cancer. During gastrulation or neural crest formation TGFβ induces motility and invasiveness, enabling cells to migrate to distant sites within the developing embryo. In contrast, in differentiated epithelial tissue TGFβ primarily maintains tissue homeostasis by inducing growth arrest and apoptosis, functioning as a tumor-suppressor (Derynck et al., 2001; Siegel and Massague, 2003; Massague, 2008).

This ambivalent nature of TGFβ signaling plays also a critical role in cancer development and progression. At early stages of tumorigenesis TGFβ functions as a tumor-suppressor by inducing cell cycle arrest and apoptosis. In contrast, during late stage tumorigenesis, TGFβ functions as an oncogene by inducing a transition of non-invasive epithelial cells into invasive mesenchymal cells, a process named epithelial-mesenchymal transition (EMT) (Roberts and Wakefield, 2003; Thiery and Sleeman, 2006; Pardali and Moustakas, 2007; Massague, 2008; Yang and Weinberg, 2008).

The hallmarks of EMT, namely the loss of epithelial markers such as E-cadherin, the gain of mesenchymal markers such as N-cadherin, the transition into mesenchymal cell shape accompanied by increased motility and invasiveness are all reminiscent of early steps of metastasis. Hence, EMT evolved to an attractive model to describe early steps of single cell-based cancer invasion and several reports identified EMT in the invasive front of human tumors (Oft et al., 1998; Rosivatz et al., 2002; Usami et al., 2008). The modulation of TGFβ signaling from a tumor-suppressor to an EMT-inducer plays an critical role during tumor progression and is achieved by two major modification namely, attenuation of the apoptotic TGFβ signaling and activation of survival ensuring, mitogenic phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways. The tumor-suppressive, canonical TGFβ signaling is mediated the by receptor associated Smad proteins (R-Smads). Upon ligand binding the R-Smads (Smad2/3) are phosphorylated, dissociate from the TGFβ-receptor complex, interact with the common mediator Smad4, translocate into the nucleus and control the expression of various genes. Cell-cycle inhibitory genes such as $p15^{INK4B}$ and $p21^{CIP1}$ are transcriptionally activated by canonical TGFβ signaling while genes such as the mitogenic c-Myc are repressed (Massague, 2004). Tumors attenuate apoptotic canonical TGFβ signaling and bypass cell-cycle arrest by repressing transforming growth factor receptor II (TGFβRII) expression or via mutational inactivation of Smad proteins (Kim et al., 2000; Osada et al., 2001; Venkatasubbarao et al., 2001; Munoz et al., 2006; Yamashita et al., 2008b). By inhibiting its tumor-suppressive function, tumor cells utilize TGFβ to drive proliferation and survival by non-canonical signaling-mediated activation of the MAPK and/ or the PI3K pathways (Gotzmann et al., 2002; Janda et al., 2002; Dumont et al., 2003;

Jechlinger et al., 2006; Lee et al., 2007). Total loss of TGFβ signaling impairs tumor progression and metastasis formation (Oft et al., 1998; Moustakas and Heldin, 2005).

Here, we introduce the homeobox transcription factor distal-less homeobox 2 (Dlx2) as a gene upregulated during EMT, which protects epithelial, non-transformed NMuMG cells from TGFβ-induced cell-cycle arrest and apoptosis. We show that Dlx2 expression is induced by canonical TGFβ signaling and functions in a negative feedback loop by inducing the transcriptional repression of the TGFβRII gene. This leads to reduced TGFβRII proteins levels, decreased Smad 4 activity and finally, to decreased expression of TGFβ target genes, such as the cell-cycle inhibitor $p21^{CIP1}$, and stabilization of the mitogenic c-Myc protein. Simultaneously, Dlx2 promotes, in cooperation with the epidermal growth factor receptor (EGFR), survival and proliferation by activating the MAPK and PI3K pathways, which are both essentially required for resistance against TGFβ-induced cell-cycle arrest and apoptosis.

3.2.3. Results

3.2.3.1. EMT induces Dlx2 expression

For the identification of genes critical in the regulation of EMT, we performed genes expression profiling and quantitative RT-PCR experiments on three independent *in vitro* EMT model systems before and after EMT, namely (i) MTdeltaECad cells, a MMTV-neu breast tumor-derived cell line, in which EMT was induced by Cre-recombinase mediated deletion of the E-cadherin gene (Lehembre et al., 2008) (ii) MFC7-shECad cells, a human breast cancer cell line, in which EMT was induced by shRNA-mediated downregulation of E-cadherin (Lehembre et al., 2008) and (iii) NMuMG cells, a murine mammary epithelial cell line, in which EMT was induced upon treatment with TGFβ (Piek et al., 1999). Screening for genes that are regulated in all three experimental systems, we identified the homeobox transcription factor Dlx2 as a commonly upregulated gene (MFC7-shECad 50x, MTdeltaECad 4x, NMuMG + TGFβ 3.5x upregulated). In NMuMG cells, Dlx2 is most upregulated after 6 days of TGFβ treatment (Figure 14A). To assess whether Dlx2 is a target of either canonical or non-canonical TGFβ signaling, we followed Dlx2 mRNA expression in

stable Smad4 knockdown NMuMG cells (shSmad4-NMuMG) treated with TGFβ (Deckers et al., 2006). In TGFβ-treated shSmad4-NMuMG cells Dlx2 expression is significantly less upregulated, indicating that Dlx2 is a target of Smad-dependent, canonical TGFβ signaling (Figure 14B). Thus, Dlx2 is upregulated during TGFβ-induced EMT as a target of canonical TGFβ signaling.

3.2.3.2. Dlx2 is not required for EMT

We next assessed whether Dlx2 expression is critical for TGFβ-induced EMT in NMuMG cells. Therefore, we transfected NMuMG cells with siRNA against Dlx2 and followed changes in cell morphology and epithelial or mesenchymal marker expression. TGFβ-treated, siDlx2-expressing NMuMG cells underwent a *bona fide* EMT, showing no phenotypic differences to control cells undergoing EMT (Figure 14C). Furthermore, expression of the epithelial marker E-cadherin and mesenchymal markers N-cadherin and NCAM were comparable to control cells, underlining that Dlx2 function is not required for EMT (Supplementary Figure S8). Interestingly, the number of surviving cells was significantly reduced in TGFβ-treated siDlx2-NMuMG cells, due to increased apoptosis and decreased proliferation (Figure 14D-F). This was confirmed by a significantly reduced cell growth of TGFβ treated NMuMG cells in which Dlx2 expression was ablated due to an shRNA-mediated knock-down (shDlx2-NMuMG) (Figure 14G). Thus, Dlx2 function is required for survival and proliferation of NMuMG cells undergoing EMT, yet not for the actual morphogenic process of EMT.

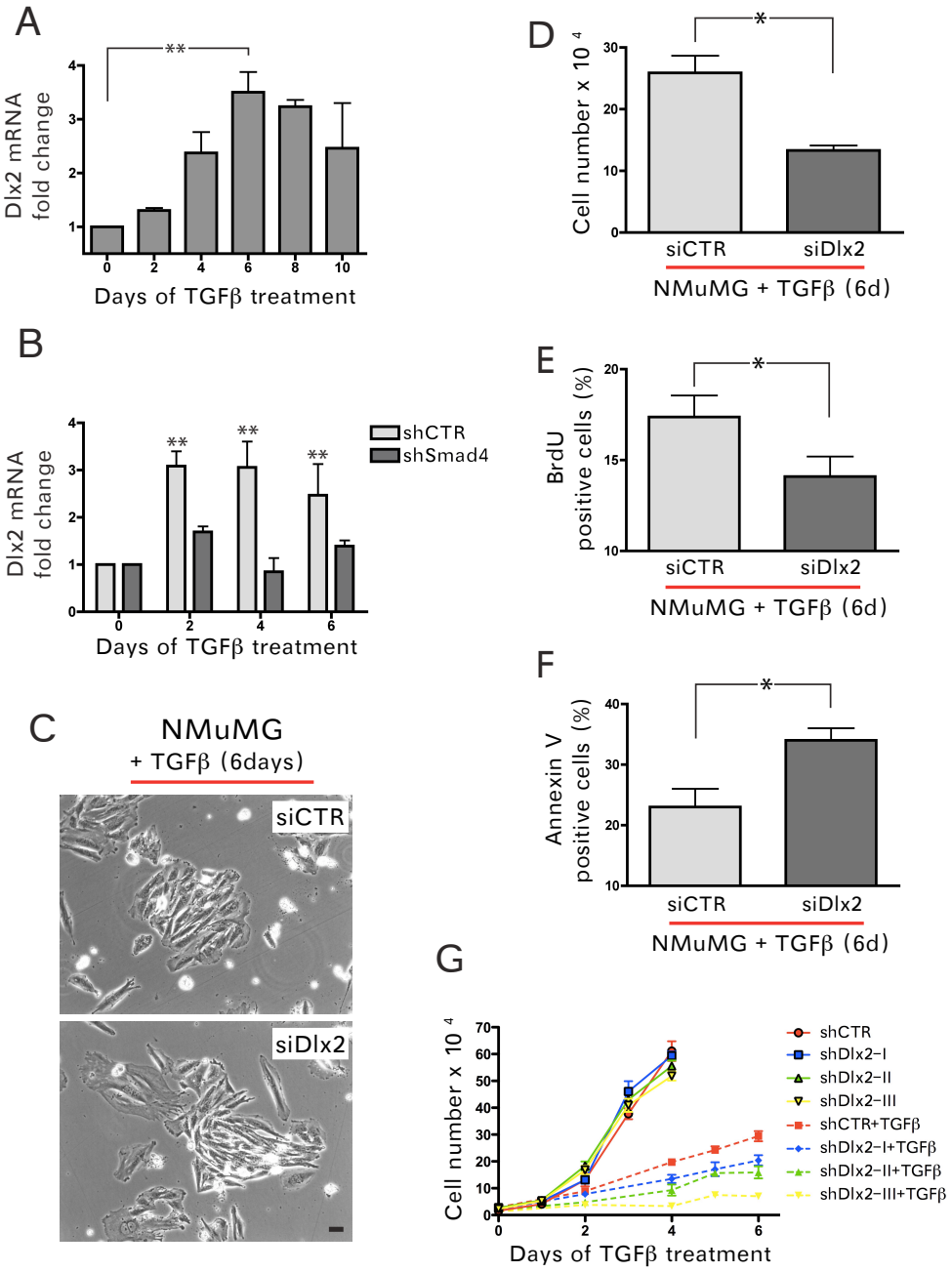


Figure 14. Dlx2 is a target of canonical TGFβ signaling and promotes survival during TGFβ-induced EMT. (A) Dlx2 is upregulated during TGFβ-induced EMT. Dlx2 mRNA levels were determined by quantitative RT-PCR in NMuMG cells treated with TGFβ for days indicated. **(B)** Dlx2 is upregulated by Smad-dependent, canonical TGFβ signaling. Dlx2 mRNA levels were determined by quantitative RT-PCR in stable Smad4 knockdown (shSmad4) and control (shCTR) NMuMG cells treated with TGFβ for days indicated. **(C)** Dlx2 function is not required for TGFβ-induced EMT. Phase-contrast microphotographs reveal a *bona fide* EMT of Dlx2-depleted (siDlx2) and control (siCTR) NMuMG cells treated with TGFβ for 6 days. **(D)** Loss of Dlx2 function reduces survival during TGFβ-induced EMT. Dlx2-depleted (siDlx2) and control

(siCTR) NMuMG cells were treated with TGFβ for days indicated. Viable cells were counted by trypan blue exclusion using a Neubauer chamber. **(E)** Loss of Dlx2 function reduces proliferation during TGFβ-induced EMT. Dlx2-depleted (siDlx2) and control (siCTR) NMuMG cells were treated with TGFβ for days indicated. Proliferation was assessed by BrdU incorporation and FACS analysis. **(F)** Loss of Dlx2 function increases apoptosis during TGFβ-induced EMT. Dlx2-depleted (siDlx2) and control (siCTR) NMuMG cells were treated with TGFβ for days indicated. Apoptosis was assessed by Annexin V staining and FACS analysis. **(G)** Loss of Dlx2 function decreases cell growth during TGFβ-induced EMT. NMuMG cells stably infected with three independent shRNA against Dlx2 (shDlx2 I-III) and control shRNA (shCTR) were treated with TGFβ for days indicated and counted using a Neubauer chamber. Data are means of three independent experiments. Statistical values are calculated by using an unpaired, two-tailed t-test. p-value ≤ 0.05 indicated with (*), p-value ≤ 0.01 indicated with (**).

3.2.3.3. Dlx2 is not sufficient to induce EMT

To investigate whether expression of Dlx2 by itself induces EMT, we stably infected NMuMG cells with lentiviral particles encoding N-terminal HA-tagged murine Dlx2 and used cell pools for further analysis. Dlx2 is exclusively expressed in the nucleus of stably infected NMuMG cells and does not induce EMT, as the cells are neither elongated nor flattened and do not reveal increased motility shown by Boyden chamber-based migration assay (Figure 15A, Supplementary Figure S9). To investigate whether expression of Dlx2 induces a loss of epithelial and a gain of mesenchymal markers, we analyzed the expression of the epithelial marker E-cadherin and the mesenchymal markers N-cadherin and NCAM. Western Blot analysis revealed a decreased expression of E-cadherin in Dlx2-expressing NMuMG cells, whereas N-cadherin and NCAM expression remained unchanged (Figure 15B). The repression of E-cadherin gene expression in Dlx2-expressing NMuMG cells was confirmed by immunofluorescence staining for E-cadherin and by the reduction in E-cadherin mRNA levels (Figure 15C, 16D). Reduced expression of E-cadherin is mediated on the transcriptional level, as shown by an E-cadherin promoter reporter assay (Figure 15D). Yet, considering that the remaining E-cadherin protein is localized in functional cell-cell junctions suggests that it is sufficient to prevent detachment and scattering of Dlx2-expressing NMuMG cells.

In summary, Dlx2-expressing NMuMG cells show a decrease in E-cadherin protein levels, but no mesenchymal morphology, increased motility nor upregulation of the mesenchymal markers N-cadherin or NCAM, thus failing to show any hallmarks of *bona fide* EMT. Thus,

Dlx2 is not sufficient to induce EMT.

3.2.3.4. Dlx2 enables TGFβ-resistant growth

We next investigated whether expression of Dlx2 affects proliferation and/or apoptosis of EMT undergoing NMuMG cells. Dlx2-expressing NMuMG cells revealed a significantly increased cell growth in comparison to control cells and displayed no sensitivity towards TGFβ-mediated growth inhibition (Figure 15E). To investigate whether this is due to increased proliferation or decreased apoptosis, we compared the rates of proliferation (BrdU incorporation) and apoptosis (Annexin V staining) of control and Dlx2-expressing NMuMG cells under TGFβ treatment. In comparison to control cells, Dlx2-expressing NMuMG cells reveal an increased proliferation and decreased apoptosis rate in presence of TGFβ (Figure 15F, G). Thus, the accelerated and TGFβ resistant growth of Dlx2-expressing NMuMG cells relies on both increased proliferation and decreased apoptosis during TGFβ-induced EMT.

In summary, loss of function and gain of function experiments confirm that Dlx2 is required for cell survival and proliferation during TGFβ-induced EMT.

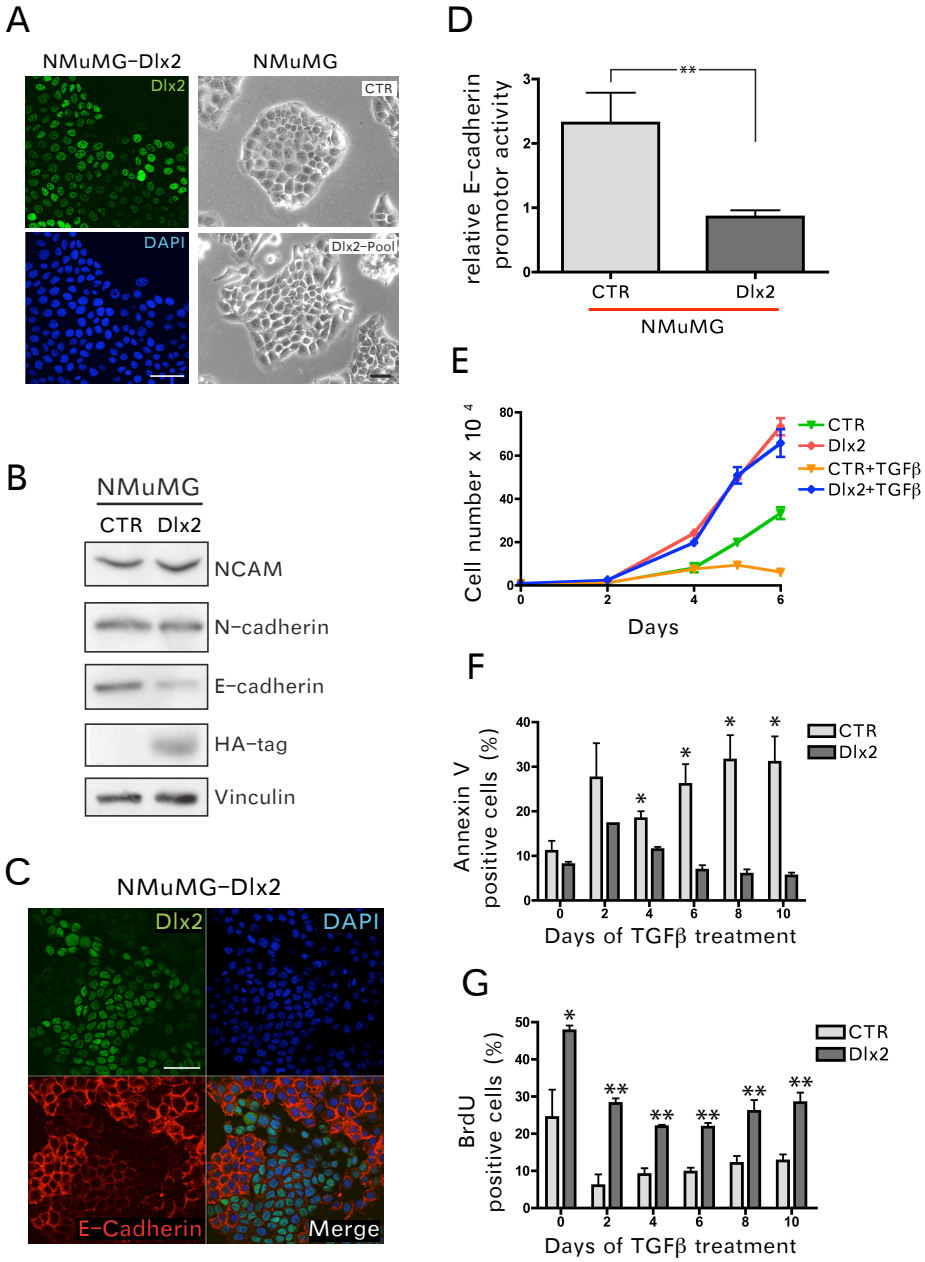


Figure 15. Dlx2 expression leads to increased proliferation and resistance towards TGFβ-induced apoptosis. (A, left panel) Dlx2 is exclusively localized in the nucleus of NMuMG cells. Confocal laser scanning microcopy NMuMG cell stably expressing N-terminal HA-tagged Dlx2 (green). Dlx2 was detected by anti-HA immunofluorescence staining. Blue DAPI staining visualizes nuclei. Size bar = 50µm. **(A, right panel)** Dlx2 expression is not sufficient to induce EMT. Phase-contrast microphotographs of Dlx2-expressing and control NMuMG cells. Note that Dlx2-expressing NMuMG cells are not individual nor flattened, showing no hallmarks of mesenchymal cells. Size bar = 50µm. **(B)** Dlx2 expression is not sufficient to increase expression of mesenchymal markers. Immunoblotting analysis for the epithelial marker E-cadherin and the mesenchymal markers NCAM and N-cadherin in Dlx2-expressing and control NMuMG cells. Immunoblotting against vinculin was used as a loading control. **(C)** Dlx2 expression reduces expression of the epithelial marker E-cadherin.

Confocal laser scanning microcopy analysis of E-cadherin in Dlx2-expressing and control NMuMG cells (red). Note that E-cadherin expression is reduced but still present in intercellular adhesions. Blue DAPI staining visualizes nuclei. Size bar = 50µm. **(D)** Dlx2 mediates transcriptional repression of the E-cadherin gene. Dlx2-expressing and control NMuMG cells were transfected with a luciferase reporter plasmid containing 423 bp of the E-cadherin promotor sequence. Values were normalized to Renilla luciferase activity. **(E)** Dlx2 expression promotes accelerated cell growth which is resistant to TGFβ-mediated growth inhibition. Dlx2-expressing and control NMuMG cells were treated with or without TGFβ for days indicated and counted using a Neubauer chamber. **(F)** Expression of Dlx2 decreases apoptosis during TGFβ-induced EMT. Dlx2-expressing and control NMuMG cells were treated with TGFβ for days indicated. Apoptosis was assessed by Annexin V staining and FACS analysis. **(G)** Expression of Dlx2 increases proliferation during TGFβ-induced EMT. Dlx2-expressing and control NMuMG cells were treated with TGFβ for days indicated.Proliferation was assessed by BrdU incorporation and FACS analysis. Data are means of three independent experiments. Statistical values are calculated by using an unpaired, two-tailed t-test. p-value ≤ 0.05 indicated with (*), p-value ≤ 0.01 indicated with (**).

3.2.3.5. Dlx2 attenuates TGFβ-induced EMT

We next investigated whether expression of Dlx2 has an impact on EMT induction and/or progression in NMuMG cells. Control and Dlx2-expressing NMuMG cells were treated with TGFβ and changes in cell morphology, invasion and molecular marker expression were followed. In contrast to control cells which exhibit a *bona fide* EMT upon TGFβ-treatment (Figure 16A, upper panel), the Dlx2-expressing NMuMG cells showed a less flattened and less elongated, but more compact cell morphology (Figure 16A, lower panel). Hence, we analyzed whether Dlx2-expressing NMuMG follow EMT-associated changes in cell adhesion and/or cytoskeletal composition. For this, we performed a immunofluorescence staining for phosphorylated focal adhesion kinase (pFAK), the intermediate filament vimentin and actin stress fibers (phalloidin). As shown, Dlx2-expressing NMuMG cells when treated with TGFβ display reduced staining for pFAK and vimentin accompanied by reduced actin-based stress fibers formation, indicating that Dlx2-expressing NMuMG cells do not undergo a *bona fide* EMT upon treatment with TGFβ. To further validate that Dlx2 expression attenuates TGFβ-induced EMT, we analyzed invasion of the control and Dlx2-expressing NMuMG cells into collagen I matrix in the presence of TGFβ. In contrast to control cells which invade into the collagen I matrix, the Dlx2-expressing NMuMG cells fail to form invasive protrusions, thus confirming that Dlx2 expression prevents EMT (Figure 16C). Next, we quantified the loss of epithelial marker and gain of mesenchymal marker expression in TGFβ-treated control and Dlx2-expressing NMuMG by quantitative RT-PCR.

The loss of the epithelial marker E-cadherin is accelerated in TGFβ-treated Dlx2-expressing NMuMG cells, whereas NCAM induction is comparable to controls (Figure 16D, E). Interestingly, Dlx2-expressing NMuMG cells reveal no upregulation of the mesenchymal marker N-cadherin (Figure 16F). In summary, Dlx2-expressing NMuMG do not undergo a *bona fide* TGFβ-induced EMT. In comparison to control cells, Dlx2-expressing cells display (i) reduced stress fibers formation (ii) reduced expression of vimentin (iii) reduced focal adhesion (iv) no formation of invasive protrusions in a 3D collagen I matrix and (v) reduced expression of mesenchymal marker N-cadherin. Moreover, although loss of E-cadherin is accelerated, NCAM induction is comparable to control cells illustrating the Dlx2 expression provokes a partial EMT.

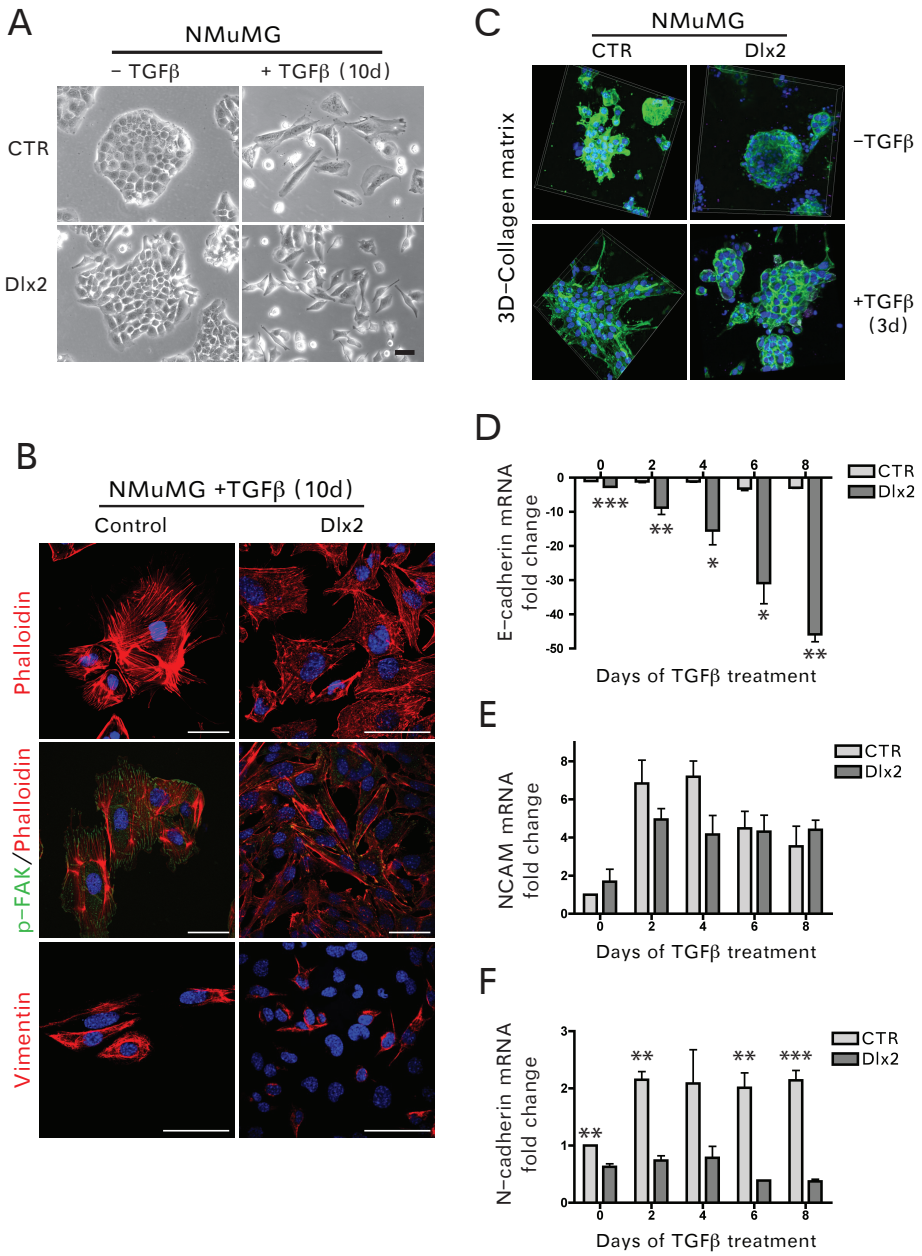


Figure 16. Dlx2-expression prevents a *bona fide* EMT. (A) Dlx2 expressing NMuMG cells do not undergo a *bona fide* EMT. Phase-contrast microphotographs of 10 days TGFβ-treated Dlx2-expressing and control NMuMG cells. Note that TGFβ-treated Dlx2-expressing cells have a less elongated and flattened cell morphology. Size bar = 50µm. **(B, upper panel)** Dlx2 expression prevents TGFβ-induced actin stress fiber formation. Confocal laser scanning microcopy of Dlx2-expressing and control NMuMG cells treated for 8 days with TGFβ. Actin fibers were visualized by phalloidin staining. Blue DAPI staining visualizes nuclei. Size bar = 50µm. **(B, middle panel)** Dlx2 expression prevents phosphorylation of focal adhesion kinase (FAK). Confocal laser scanning microcopy of Dlx2-expressing and control NMuMG cells treated for 8 days with TGFβ. Phosphorylated FAK was visualized by immunostaining (green), actin stress fiber by phalloidin staining (red). Blue DAPI staining visualizes nuclei. Size bar = 50µm. **(B, lower panel)** Dlx2 expression prevents upregulation of the

mesenchymal intermediate filament vimentin. Confocal laser scanning microcopy of Dlx2-expressing and control NMuMG cells treated for 8 days with TGFβ. Vimentin was visualized by immunostaining (red) and vimentin positive cells were counted: CTR-NMuMG cells were 96%, Dlx2-expressing NMuMG cells were 26% positive for vimentin. Size bar = 50µm. **(C)** Dlx2 expression prevents EMT-mediated invasion into collagen I. Dlx2-expressing and control NMuMG cells were embedded in collagen I and treated with or without TGFβ for 3 days. Actin cytoskeleton was stained by phalloidin (green) and visualized by confocal laser scanning microcopy. **(D)** Dlx2 expression accelerates loss of E-cadherin expression during TGFβ-induced EMT. E-cadherin mRNA levels were determined by quantitative RT-PCR in NMuMG cells treated with TGFβ for times indicated. Values were normalized to endogenous RPL19 levels. **(E)** Dlx2 expression does not prevent induction of NCAM during TGFβ-induced EMT. NCAM mRNA levels were determined by quantitative RT-PCR in NMuMG cells treated with TGFβ for times indicated. Values were normalized to endogenous RPL19 levels. **(F)** Dlx2 expression prevents upregulation of N-cadherin during TGFβ-induced EMT. N-cadherin mRNA levels were determined by quantitative RT-PCR in NMuMG cells treated with TGFβ for times indicated. Values were normalized to endogenous RPL19 levels. Data are shown as mean ± SD and are representative of three independent experiments. Statistical values are calculated by using an unpaired, two-tailed t-test. p-value ≤ 0.05 indicated with (*), p-value ≤ 0.01 indicated with (**), p-value ≤ 0.001 indicated with (***).

3.2.3.6. Dlx2 inhibits canonical TGFβ signaling

Tumor cells are well known to escape from TGFβ-mediated growth inhibition by attenuating the apoptotic, canonical TGFβ-signaling. This is shown to be achieved by repression of the TGFβ receptor II (TGFβRII) levels or by inactivating mutations of Smad proteins (Chakravarthy et al., 1999; Gobbi et al., 1999; Gobbi et al., 2000; Buck et al., 2004). To investigate whether attenuation of the apoptotic, canonical TGFβ signaling pathway is responsible for the TGFβ resistant cell growth of Dlx2-expressing NMuMG cells, we determined the presence and activities of different canonical TGFβ signaling molecules. Protein levels of the TGFβRII were decreased in Dlx2-expressing NMuMG cells, whereas Smad4 protein levels were unchanged (Figure 17A). Decreased TGFβRII promoter reporter activity accompanied by reduced TGFβRII mRNA levels illustrated that repression of TGFβRII is mediated on the transcriptional level (Figure 17B, C). Consequently, following the reduction of TGFβRII protein levels, phosphorylation of the effector protein Smad2 was reduced along with the activity of the common mediator Smad4, as illustrated by western blot and decreased Smad4 luciferase reporter activity (CAGA box reporter), respectively (Figure 17D, E). Finally, decreased TGFβ signaling lead to reduced transcriptional control of TGFβ target proteins, as shown by reduced expression of the cyclin-dependent kinase inhibitor $p21^{CIP1}$ and stabilization of the mitogenic c-Myc protein (Figure 17F, 18A lower panel). We

found no upregulation of inhibitory Smads such as Smad7 nor upregulation of the Smad specific E3 ubiquitin protein ligase 1 (Smurf1) which were shown to inhibit TGFβ receptor signaling and promote TGFβ receptor degradation, respectively (Data not shown) (Di Guglielmo et al., 2003; Zhang et al., 2007).

In summary, expression of Dlx2 leads to a transcriptional repression of the TGFβRII gene, reduced Smad activity, reduced expression of the cell-cycle inhibitor $p21^{CIP1}$ and stabilization of the mitogenic c-Myc protein, thus inhibiting the apoptotic, canonical TGFβ signaling pathway..

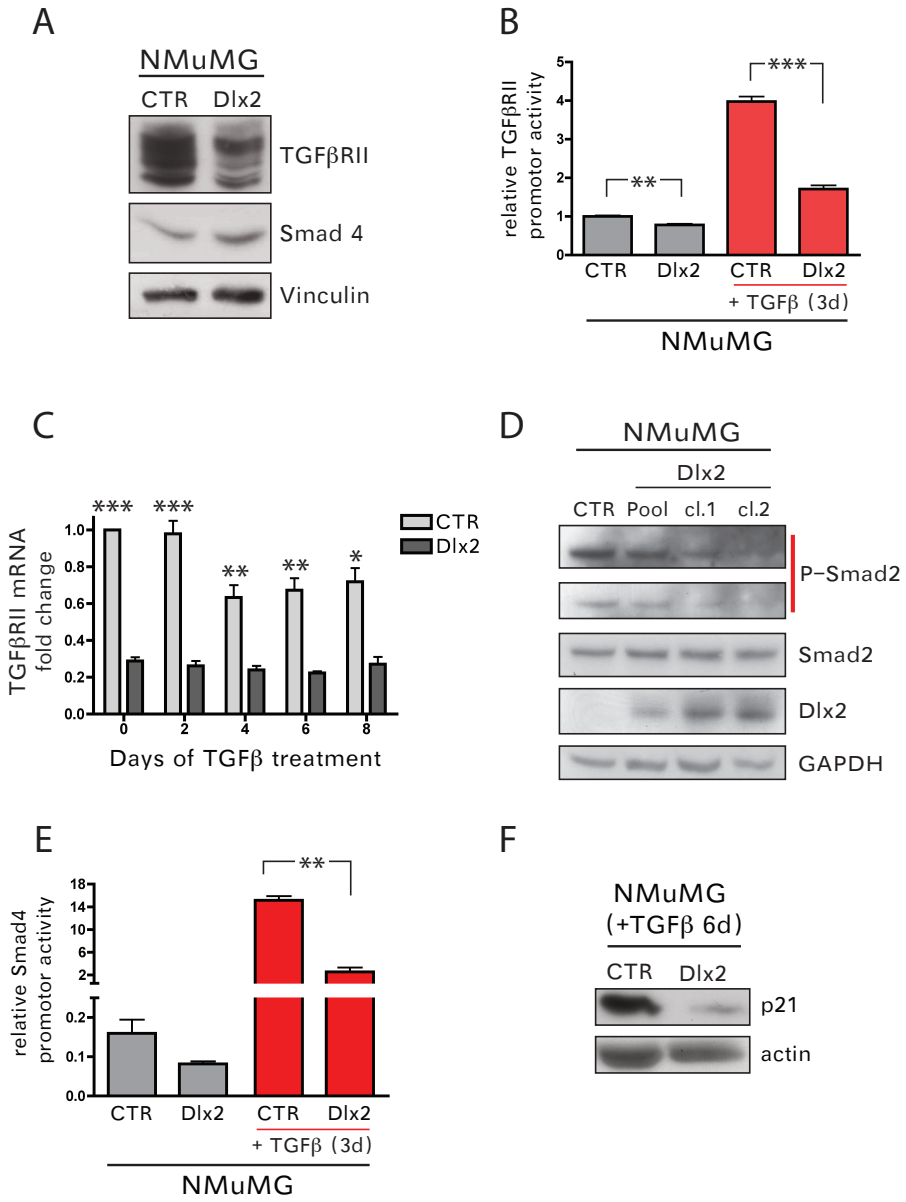


Figure 17. Dlx2 expression attenuates apoptotic, canonical TGFβ signaling. (A) Dlx2 expression reduces TGFβRII expression in NMuMG cells. Immunoblotting analysis of Dlx2-expressing and control NMuMG cells. Immunoblotting against vinculin was used as a loading control. **(B)** Dlx2 mediates transcriptional repression of the TGFβRII gene. Dlx2-expressing and control NMuMG cells were transfected with a luciferase reporter plasmid containing 255 bp of the TGFβRII promotor sequence and treated with or without TGFβ for 3 days. Values were normalized to Renilla luciferase activity.**(C)** Dlx2 expression reduces TGFβRII mRNA levels in NMuMG cells. TGFβRII mRNA levels were determined by quantitative RT-PCR. Values were normalized to endogenous RPL19 levels. **(D)** Dlx2 expression provokes reduced phosphorylation of Smad2 proteins. Immunoblotting analysis of Dlx2-expressing (Pools and Dlx2 high expressing clones 1, 2) and control NMuMG cells. Immunoblotting against GAPDH was used as a loading control. **(E)** Dlx2 expression in NMuMG cells decreases Smad 4 activity. Dlx2-expressing and control NMuMG cells were transfected with a luciferase reporter plasmid

containing repetitive elements of Smad4 binding motifs and treated with or without TGFβ for 3 days. Values were normalized to Renilla luciferase activity. **(F)** Dlx2 expression decreaeses expression of the cell cycle inhibitor $p21^{CIP1}$. Immunoblotting analysis of Dlx2-expressing and control NMuMG cells treated for 6 days with TGFβ. Immunoblotting against actin was used as a loading control. Data are shown as mean ± SD and are representative of three independent experiments. Statistical values are calculated by using an unpaired, two-tailed t-test. p-value ≤ 0.05 indicated with (*), p-value ≤ 0.01 indicated with (**), p-value ≤ 0.001 indicated with (***).

3.2.3.7. Dlx2 promotes MAPK and PI3K activation

The MAPK and PI3K pathways are well known to be interactively engaged upon TGFβ signaling to ensure survival and proliferation (Janda et al., 2002; Lee et al., 2007). Hence, we investigated if these pathways were also active in Dlx2-expressing NMuMG cells. In order to follow MAPK activity we analyzed the phosphorylation of the MAPK Erk1/2. As shown, Erk1/2 phosphorylation levels are constantly higher in Dlx2-expressing NMuMG cells in the absence as well as presence of TGFβ (Figure 18A). Assessing whether increased MAPK signaling is responsible for the accelerated and TGFβ-resistant growth of Dlx2-expressing NMuMG cells, we treated both control and Dlx2-expressing NMuMG cells with an inhibitor for the MAPKK MEK1/2 (PD98059) and quantified cell growth in the absence or presence of TGFβ. Treatment with PD98059 for 4 days lead to an increased growth reduction of Dlx2-expressing NMuMG cells (-54%) as compared to control cells (-20%), indicating that Dlx2-mediated accelerated cell growth relies partially on MAPK activity (Figure 18B). Notably, PD98059 treatment of Dlx2-expressing NMuMG cells did not normalize cell growth to control levels, indicating that MAPK activity was not solely responsible for Dlx2-mediated accelerated proliferation. Combined treatment with TGFβ and PD98059 for 4 days lead to further growth reduction of Dlx2-expressing NMuMG cells (-60%), whereas control cell growth completely collapsed (-96%) (Figure 18B). Thus, increased MAPK activity is important for the accelerated cell growth of Dlx2-expressing NMuMG cells but is not essential for TGFβ resistant proliferation.

To investigate whether the PI3K pathway is cooperating with the MAPK pathway to ensure accelerated and TGFβ resistant cell growth of Dlx2-expressing NMuMG cells, we assessed the phosphorylation of the PI3K target protein kinase B (PKB). PKB is increased in phosphorylation in Dlx2-expressing NMuMG cells on both activation sites (Thr308 and

Ser473) in the presence as well as absence of TGFβ (Figure 18A). To investigate to what extent PI3K signaling is required for TGFβ resistant growth of Dlx2-expressing NMuMG cells, we treated both control and Dlx2-expressing NMuMG cells with the PI3K inhibitor ZSTK474 for 4 days in the presence or absence of TGFβ and determined cell growth. In contrast to control cells whose growth slightly declines after 4 days treatment with ZSTK474 (-10%), the growth of Dlx2-expressing NMuMG cells was strongly reduced and almost normalized to control cell growth (-61%) (Figure 18C). Furthermore, combined treatment with TGFβ and ZSTK474 for 4 days resulted in further significant growth reduction of both control (-88%) and Dlx2-expressing NMuMG cells (-76%) (Figure 18C). Thus, comparable to MAPK signaling, the PI3K plays an important role for Dlx2-mediated acceleration of growth, but is not solely responsible for TGFβ-resistant proliferation. Comparing the magnitude of inhibition mediated by either PD98059 and ZSTK474, we assume that PI3K activity plays a pivotal role in Dlx2-mediated TGFβ resistant growth. In summary, Dlx2-mediated TGFβ-resistant cell growth relies on the cooperative activity of the MAPK and the PI3K pathways.

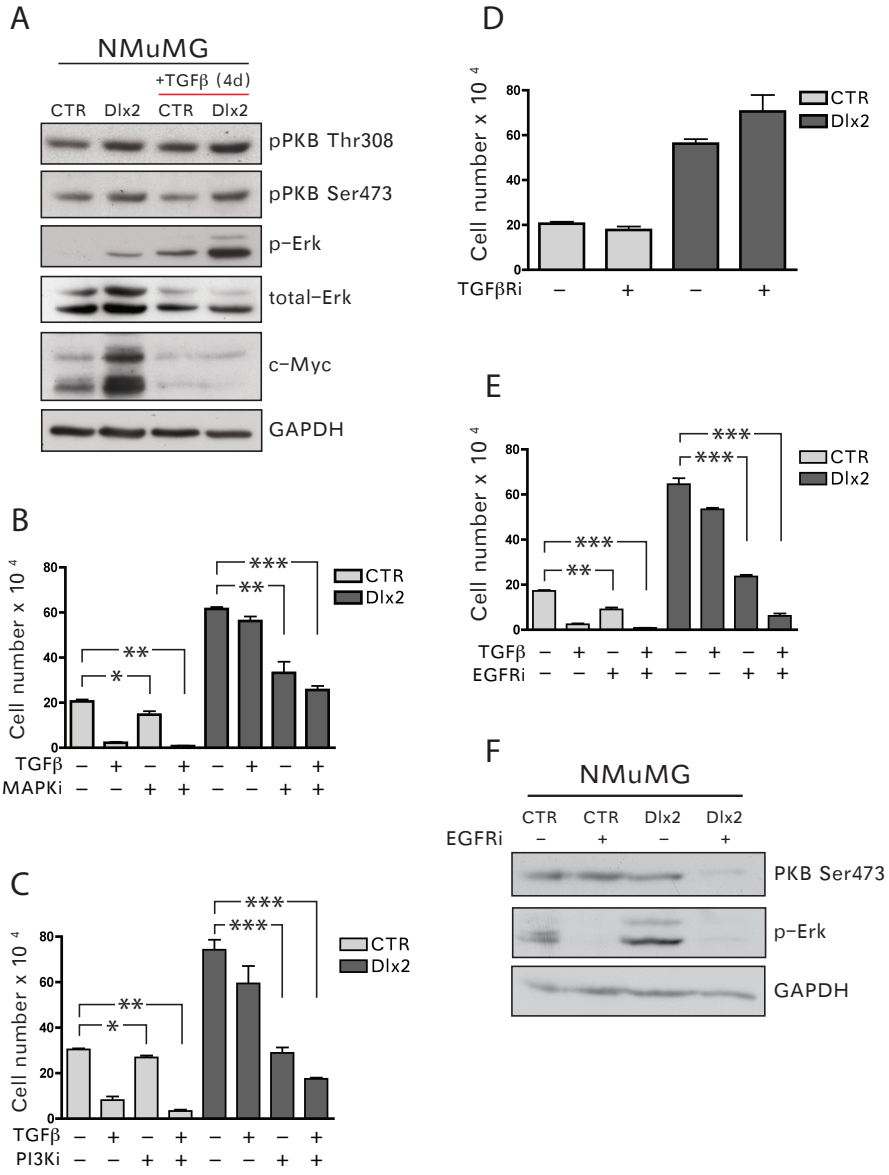


Figure 18. Dlx2 promotes resistance against TGFβ-mediated growth inhibition via cooperated activation of the MAPK and PI3K signaling pathways. (A) Dlx2 expression increases phosphorylation of the MAPK Erk1/2 and the PI3K target protein PKB at both activation sites (Serine473/Threonine308). Immunoblotting analysis of Dlx2-expressing and control NMuMG cells treated with or without TGFβ for 4 days. Immunoblotting against GAPDH was used as a loading control. **(B)** Dlx2-mediated TGFβ-resistant growth relies on MAPK activity. Dlx2-expressing and control NMuMG cells were treated with TGFβ for 4 days in combination with the MAPK inhibitor PD98059 (9.35µM) or DMSO (solvent control) and counted using a Neubauer chamber. **(C)** Dlx2-mediated TGFβ-resistant growth relies on PI3K activity. Dlx2-expressing and control NMuMG cells were treated with TGFβ for 4 days in combination with the PI3K inhibitor ZSTK474 (0.239µM) or DMSO (solvent control) and counted using a Neubauer chamber. **(D)** Dlx2-mediated increased proliferation does not depend on an autocrine TGFβ-loop. Dlx2-expressing and control NMuMG cells were treated with the TGFβRI inhibitor SB431542 (3µM) or DMSO (solvent control) and counted using a Neubauer chamber. **(E)** Dlx2-mediated TGFβ-resistant

growth relies on EGFR activity. Cells were treated with TGFβ for 4 days in combination with the EGFR inhibitor AG1478 (0.775 µM) or DMSO (solvent control) and counted using a Neubauer chamber. **(F)** Inhibition of EGFR activity reduces MAPK Erk1/2 and PKB phosphorylation in Dlx2-expressing NMuMG cells. Immunoblotting analysis of Dlx2-expressing and control NMuMG cells treated with the EGFRi AG1478 (1.55µM) for 6 hours. Immunoblotting against GAPDH was used as a loading control. Data are shown as mean ± SD and are representative of three independent experiments. Statistical values are calculated by using an unpaired, two-tailed t-test. p-value ≤ 0.05 indicated with (*), p-value ≤ 0.01 indicated with (**), p-value ≤ 0.001 indicated with (***).

3.2.3.8. Dlx2 engages EGFR for TGFβ resistant growth

Invasive tumor cells are known to promote growth and invasion by utilizing TGFβ within an autocrine, positive feedback loop (Park et al., 2000; Dumont et al., 2003). This is known to be achieved by activation of the MAPK and/or PI3K pathways through non-canonical TGFβ signaling (Lee et al., 2007, Bakin et al., 2000). Hence, we assessed whether Dlx2-mediated accelerated growth was driven by an autocrine, positive feedback loop of TGFβ. Control and Dlx2-expressing NMuMG cells were treated with an inhibitor for TGFβRI (SB431542) and cell growth was quantified. Treatment of both control or Dlx2-expressing NMuMG cells with SB431542 for 4 days did not reveal any significant difference in cell proliferation, revealing that the accelerated growth of Dlx2-expressing NMuMG cells is not caused by an autocrine TGFβ loop (Figure 18D).

ErbB family members are known to prevent TGFβ-induced cell-cycle arrest and apoptosis by activating the PI3K pathway (Fabregat et al., 1996; Fabregat et al., 2000; Murillo et al., 2005; Del Castillo et al., 2006). Furthermore, neuronal transit-amplifying cells, which were positive for Dlx2, were shown to be highly responsive to epidermal growth factor (EGF) stimulation. In developing olfactory bulb interneurons, Dlx1 and Dlx2 were shown to control the expression of ErbB4, indicating that Dlx2 could engage ErbB family members to drive proliferation and survival during TGFβ-mediated selection (Doetsch et al., 2002; Long et al., 2007). Therefore, we investigated whether inhibition of EGFR signaling impaired accelerated, TGFβ-resistant growth of Dlx2-expressing NMuMG cells. For this purpose, we treated control and Dlx2-expressing NMuMG cells with the EGFR inhibitor AG1478 and assessed cell growth. Treatment with AG1478 for 4 days lead to an reduced growth of Dlx2-expressing NMuMG cells (-54%) as compared to control cells (-20%), indicating that Dlx2-

mediated accelerated cell growth relied on EGFR activity (Figure 18E). Combined treatment with the AG1478 and TGFβ for 4 days induced growth arrest and apoptosis of both control cells (-97%) and Dlx2-expressing NMuMG cells (-90%), demonstrating that Dlx2-mediated TGFβ resistant growth relied on the EGFR activity (Figure 18E). Interestingly, the magnitudes of growth inhibition mediated by AG1478 in the presence as well as in the absence of TGFβ resembled ZSTK474-mediated PI3K inhibition, pointing towards a functional relationship between EGFR and PI3K activity. To address whether inhibition of the EGFR affects MAPK and/or PI3K signaling, we analyzed the phosphorylation of the MAPK Erk1/2 and PKB in control and Dlx2-expressing NMuMG cells after AG1478 treatment. Treatment of Dlx2-expressing NMuMG cells with AG1478 lead to reduced phosphorylation of PKB and Erk1/2, thus showing that EGFR is required for the activation of both pathways (Figure 18F). To confirm that EGF induces PI3K and MAPK activation, we stimulated starved control and Dlx2-expressing NMuMG cells with recombinant EGF (rEGF) and followed Erk1/2 and PKB phosphorylation. Treatment of starved Dlx2-expressing NMuMG cells with rEGF lead to increased and sustained phosphorylation of PKB and MAPK Erk1/2 (Supplementary Figure S10).

Thus, EGFR activation leads to increased PI3K and MAPK activity in Dlx2-expressing NMuMG cells which is required for survival and proliferation during TGFβ-induced EMT.

3.2.3.9. Dlx2 is required for tumor growth and metastasis

EMT and resistance towards TGFβ-mediated growth inhibition plays an important role in tumor progression and metastasis formation of melanomas (Silye et al., 1998; Sanders et al., 1999; Poser et al., 2001; Teicher, 2001; Kuphal and Bosserhoff, 2006; Boone et al., 2008). Hence, we investigated whether knockdown of Dlx2 expression in B16 melanomas cells impairs their ability to form a tumor and to metastasize. First, we analyzed whether Dlx2 is a target of TGFβ signaling in melanoma cells, by treating B16 melanoma cells for 4 days with TGFβ and assessing Dlx2 expression. Dlx2 mRNA is 6x upregulated in 4 days TGFβ-treated

B16 melanomas, confirming that Dlx2 is a target of TGFβ signaling (Figure 19A). Next we investigated whether knockdown of Dlx2 impairs the ability of B16 melanoma cells to form a tumor and to metastasize, by performing subcutaneous injections of three independent stable Dlx2 knockdown B16 melanoma cells (shDlx2-B16 I-III) into C57BL/6 mice and quantifying tumor growth and metastasis to the lung. Knockdown of Dlx2 in B16 melanomas cells resulted in a significant reduction in primary tumor growth, showing that Dlx2 played an important role in primary tumor formation (Figure 19B). To investigate whether the potential to metastasize is impaired in shDlx2-B16 melanomas, we quantified micro-metastatic lesions in the lung of C57BL/6 mice injected subcutaneously with shDlx2-B16 melanoma cells. The number of micro-metastatic lesion was significantly decreased in C57BL/6 mice injected with shDlx2-B16 melanoma cells, showing that Dlx2 expression was important for the metastatic spread of B16 melanoma cells (Figure 19C, D). In summary, suppression of Dlx2 in B16 melanoma cells resulted in decreased primary tumor growth and metastasis formation, indicating that Dlx2 is required for efficient tumor growth and metastasis formation.

Next we investigated whether increased expression of Dlx2 correlated with the ability of human tumors to grow, progress and to metastasize. Surveying gene expression profiles of human cancer biopsies using the NextBio.com database, we found a positive and significant correlation of increased Dlx2 expression and the potential of melanoma and lung cancer to metastasize. (Table 1). In prostate and lung cancer, also a positive and significant correlation of increased Dlx2 expression and advances in tumor stage was detected, indicating that Dlx2 may play a significant role in primary tumor formation (Table 1). Treatment of human glioma cells with an inhibitor for TGFβRI results in a significant downregulation of Dlx2, indicating that Dlx2 is also a target of TGFβ signaling in gliomas and could have an impact on tumor growth and metastasis formation (Table 1). Interestingly, for all these tumors, in particular in melanomas, it is well known that the resistance towards TGFβ-mediated growth inhibition and EMT plays an essential role for their metastatic spread, supporting the hypothesis that Dlx2 has a protective function during human tumor-progression and metastasis formation .

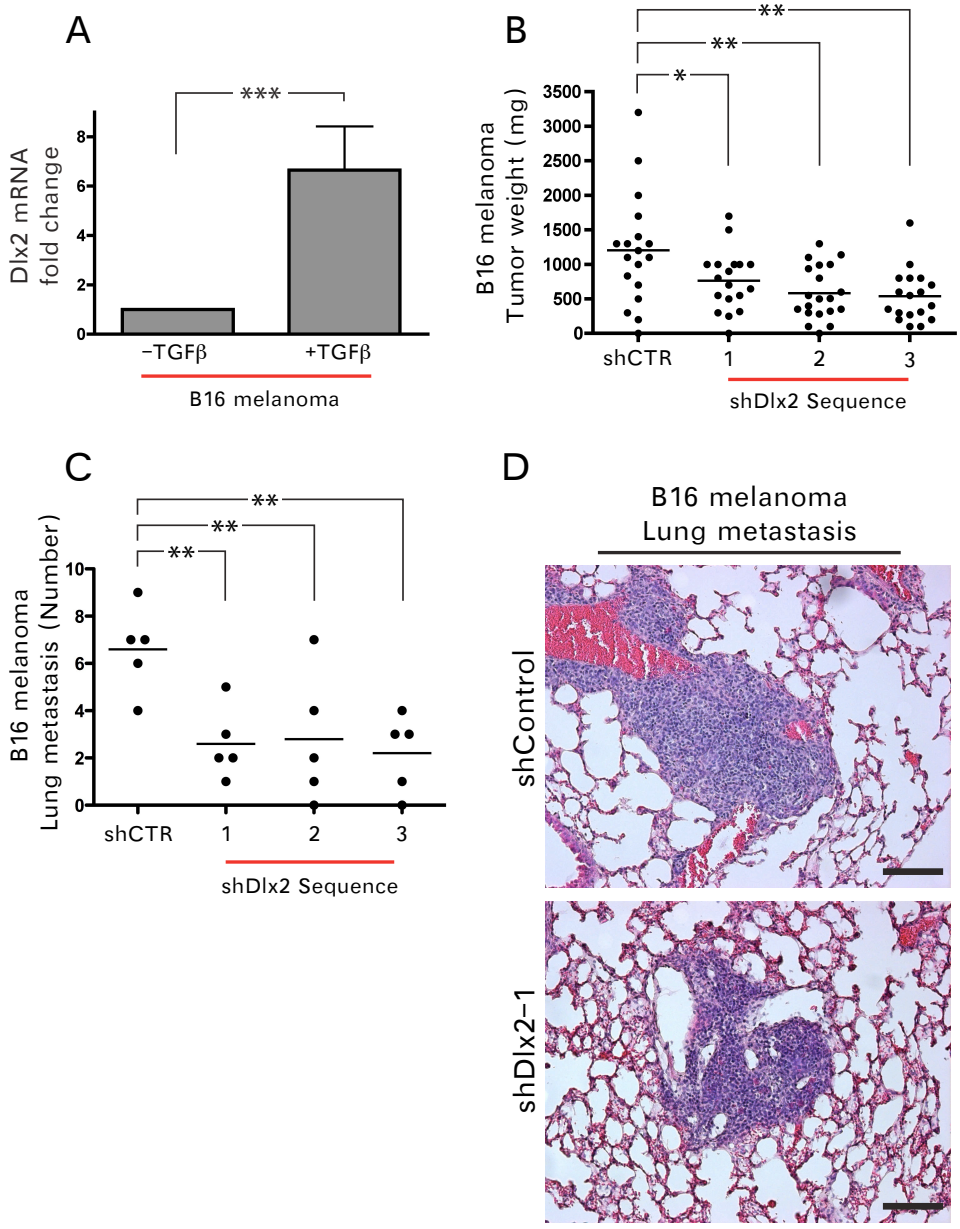


Figure 19. Dlx2 is required for primary tumor growth and metastasis to the lung. (A) Treatment of B16 melanomas cells with TGFβ induces upregulation of Dlx2. B16 melanoma cells were treated with TGFβ for 6 days and Dlx2 mRNA levels were determined by quantitative RT-PCR. Values were normalized to endogenous RPL19 levels. **(B)** Loss of Dlx2 function impairs tumor growth of B16 melanoma cells. B16 melanoma cells were ablated for Dlx2 by shRNA mediated knockdown using three independent Dlx2-specific shRNA sequences (shDlx2 1-3). Cells were injected into both flanks in C57/Bl6 mice and tumor weight was measured after 2 weeks of incubation time. N=18. **(C)** Loss of Dlx2 function impairs metastatic potential of B16 melanoma cells. B16 melanoma cells were ablated for Dlx2 by shRNA mediated knockdown using three independent Dlx2-specific shRNA sequences (shDlx2 1-3). Cells were injected into both flanks in C57/Bl6 mice and micrometastatic lesions in the lung were counted after 2 weeks of incubation time. **(D)** Micrometastatic lesion in the

lung of C57/Bl6 mice. Serial histological sections of lungs from C57/Bl6 injected subcutaneously with shDlx2-1 and shControl B16 melanomas cells were stained with hematoxylin/eosin. N=5. Size bar = 100 µm. Data are shown as mean ± SD and are representative of three independent experiments. Statistical values are calculated by using an unpaired, two-tailed t-test. p-value ≤ 0.05 indicated with (*), p-value ≤ 0.01 indicated with (**), p-value ≤ 0.001 indicated with (***).

Cancer type	Bioset name	p-value	fold upregulation	NCBI-GEO accession number
Melanoma tumors	Intermediate metastatic potential melanoma vs. Foreskin melanocyte normal	0.0451	4.19	GSE4845
	High metastatic potential melanoma vs. Foreskin melanocyte normal	0.0361	4.93	GSE4845
	Intermediate metastatic potential melanoma vs. Low metastatic potential melanoma	0.0196	2.52	GSE4845
	High metastatic potential melanoma vs. Low metastatic potential melanoma	0.0028	3.03	GSE4845
	High metastatic potential melanoma vs. Low metastatic potential melanoma	0.0067	2.96	GSE4845
	Metastatic melanoma vs. normal melanocytes	0.0021	18.8	GSE4570
Lung cancer	Stage 4 vs. Stage 1	0.0123	3.847	GSE2109
	Distant metastasis vs. no metastasis	0.0064	3.045	GSE2109
	Stage 4 vs. Stage 1	0.012	3.087	GSE2109
	Stage 2A vs. Stage 1b	0.0351	3.29	GSE3141
Prostate cancer	State 4 vs. stage 2	0.0039	3.27	GSE6919
Glioma	TGFβ inhibition leads to Dlx2 downregulation	0.0037	-21.9	(Bruna et al., 2007)

Table 1. Dlx2 expression in human cancers. Expression of Dlx2 correlates significantly with advanced tumor progression and metastatic potential of melanoma, glioma, lung and prostate cancers. Microarray data are accessible at NCBI Gene Expression Omnibus (GEO) database.

3.2.4. Discussion

Overcoming TGFβ-mediated growth inhibition at early stages of tumor development and to use it at later stages as a pro-invasive factor, plays an essential role in tumor progression and metastasis formation (Roberts and Wakefield, 2003; Thiery and Sleeman, 2006; Pardali and Moustakas, 2007; Massague, 2008; Yang and Weinberg, 2008). Hence, to understand the molecular mechanisms enabling cancer cells to prevail TGFβ-induced cell-cycle arrest and apoptosis plays an important role in cancer therapy. *In vitro* EMT models, such as the non-transformed NMuMG cells used here, provide experimental systems which can be used to dissect to molecular mechanism promoting resistance against TGFβ-mediated growth arrest and EMT.

In the present study, we performed gene expression profiling experiments and quantitive RT-PCR analysis of different *in vitro* EMT model systems to identify the transcription factor Dlx2 as a commonly upregulated gene during EMT. By utilizing the established NMuMG cells as an TGFβ-induced EMT-model system, the B16 mouse melanoma model and human cancer gene expression databases we identified Dlx2 as a transcription factor promoting tumor growth and metastasis.

Dlx2 is upregulated by canonical TGFβ signaling and loss of function as well as gain of function studies in NMuMG cells revealed that Dlx2 function is not required and its expression is not sufficient to induce EMT. Instead, we found that Dlx2 function is crucial for the protection from TGFβ-induced cell-cycle arrest and apoptosis. This is achieved by two major modifications, namely the inhibition of the apoptotic TGFβ signaling pathway and the simultaneous activation of the mitogenic and survival-ensuring MAPK and PI3K pathways, respectively (Figure 20):

The Dlx2-mediated attenuation of the canonical TGFβ signaling pathway is a consequence of a transcriptional repression of the TGFβ receptor II (TGFβRII) gene, leading to reduced TGFβRII protein levels, decreased activation of the signal transducers Smad2/4 and thus reduced transcriptional repression and/or activation of TGFβ target genes, such as the mitogenic c-Myc protein or the cell-cycle inhibitor $p21^{CIP1}$, respectively. Whether Dlx2 binds

directly to the TGFβRII promotor to repress its transcription remains elusive. Since no consensus binding motif for Dlx2 has been defined, we was not possible to perform any *in silico* studies. The Dlx2-driven mitogenic and proliferative signals are mediated by the EGFR, which activates the MAPK and the PI3K pathways to guarantee survival and proliferation in the presence of TGFβ (Figure 20). This goes in line with studies showing that EGF protects from TGFβ-induced apoptosis by stimulating PI3K activity and that neuronal transit amplifying cells, which are overexpressing Dlx2, are found to be highly responsive to epidermal growth factor (EGF) stimulation (Doetsch et al., 2002; Murillo et al., 2005; Del Castillo et al., 2006; Uttamsingh et al., 2008). We found no upregulation of the EGFR nor increased production of its ligand EGF in Dlx2-expressing NMuMG cells. Hence, the mechanism how Dlx2 triggers EGFR signaling to promote survival and proliferation remains elusive and is under current investigations in our laboratory.

As a tribute to reduced canonical TGFβ signaling, Dlx2-expressing NMuMG cells do not undergo a *bona fide* TGFβ-induced EMT, as the cells reveal no typical mesenchymal cell morphology, no formation of stress fiber, no invasion into collagen and less upregulation of mesenchymal markers like N-cadherin or vimentin. Still, the loss of E-cadherin is accelerated in Dlx2-expressing NMuMG cells and NCAM induction is comparable to TGFβ-treated control cells. Thus, depending on the expression levels of Dlx2, cells may undergo only a partial EMT, which could explain why the detection of full EMT is rare in human cancer biopsies (Tarin et al., 2005; Thompson et al., 2005).

Supporting the importance of Dlx2 function during tumor development and progression, we performed loss of function studies by generating stable Dlx2 knockdown B16 melanoma cells which were subcutaneously injected into syngeneic C57/Bl6 mice. Quantifying primary tumor growth and micrometastatic lesions in the lung, we found that loss of Dlx2 function significantly impairs the ability of B16 melanomas to form primary tumors and metastasis in the lung. Furthermore, surveying gene expression database of human cancer biopsies have revealed that Dlx2 expression significantly correlates with tumor progression and the metastatic potential of human melanoma, prostate and lung cancers. Interestingly, for these cancers the resistance towards TGFβ-mediated growth inhibition plays an essential role

during tumor progression and metastasis formation (Heredia et al., 1996; Guo et al., 1997; Derynck et al., 2001; Lucke et al., 2001; Buck et al., 2004; Ao et al., 2006; Hoek et al., 2006; Biswas et al., 2007; Borczuk et al., 2008; Jeon et al., 2008).

In summary, we identified the transcription factor Dlx2 as an upregulated target gene of canonical TGFβ signaling, which functions in a negative feedback loop to attenuate apoptotic TGFβ signaling, thereby enabling cells to survive and to undergo EMT. The correlation between the gain of Dlx2 expression and the metastatic capacity of human and murine tumors indicate that Dlx2 has a critical function in malignant tumor progression and metastasis and, thus, warrant further investigation.

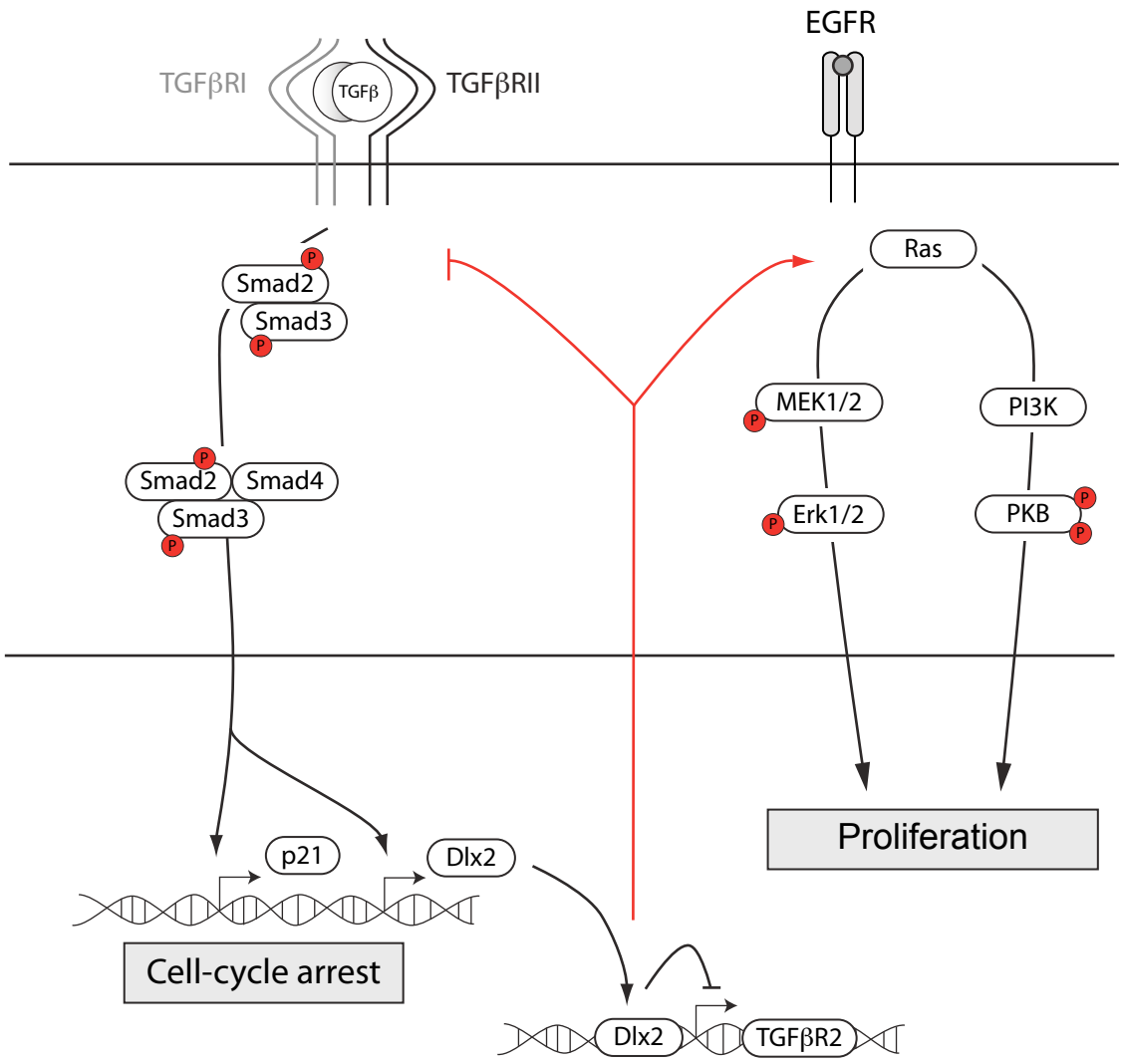
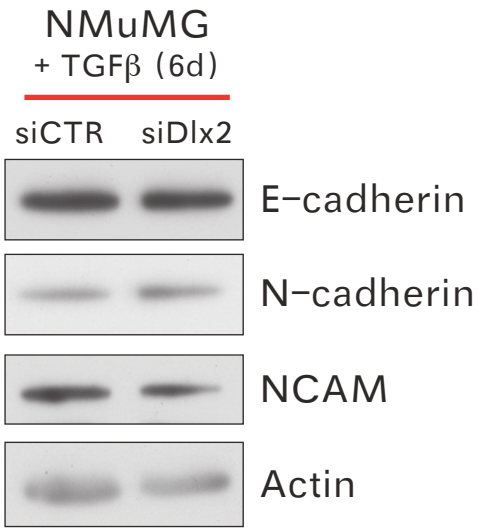


Figure 20. Dlx2 upregulation protects from TGFβ induced cell-cycle arrest and apoptosis. A working model of the molecular mechanisms underlying Dlx2-mediated protection from TGFβ-induced cell-cycle arrest and apoptosis is depicted. Binding of TGFβ to the TGFβ receptor induces phosphorylation and activation of the receptor associated signal transducers Smad2/3. Activated Smad2/3 forms with Smad4 a trimeric complex and enters the nucleus to induce expression of TGFβ-target genes such as the cell cycle inhibitor $p21^{CIP1}$ or the transcription factor distal-less homeobox 2 (Dlx2). Dlx2 expression promotes the transcriptional repression of the TGFβRII gene (dashed lines). This leads to reduced TGFβ receptor expression, reduced Smad2/3 activation and decreased expression of cytostatic genes such as $p21^{CIP1}$. Simultaneously, Dlx2 promotes epidermal growth factor receptor (EGFR) mediated activation of the mitogenic and pro-survival MAPK and PI3K pathways, leading to MAPK Erk1/2 and PKB phosphorylation, respectively. Thus, the transcription factor Dlx2 ensures survival during TGFβ-induced EMT by attenuating the apoptotic TGFβ signaling pathway and by activating mitogenic and survival promoting MAPK and PI3K pathways, respectively.

3.2.5. Supplementary Figures



Supplementary Figure S8: Dlx2 is not required for EMT. Immunoblotting analysis for the epithelial marker E-cadherin and the mesenchymal markers N-cadherin and NCAM in Dlx2-depleted (siDlx2) and control (siCTR) NMuMG cells treated with TGFβ for 6 days. Immunoblotting against GAPDH was used as a loading control.

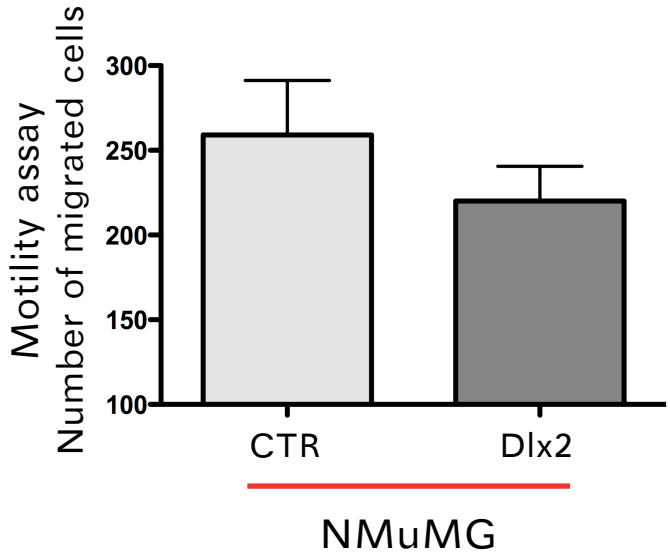


Figure S9: Dlx2 expression does not increase motility of NMuMG cells. Boyden chamber-based motility assay of Dlx2-expressing and control NMuMG cells.

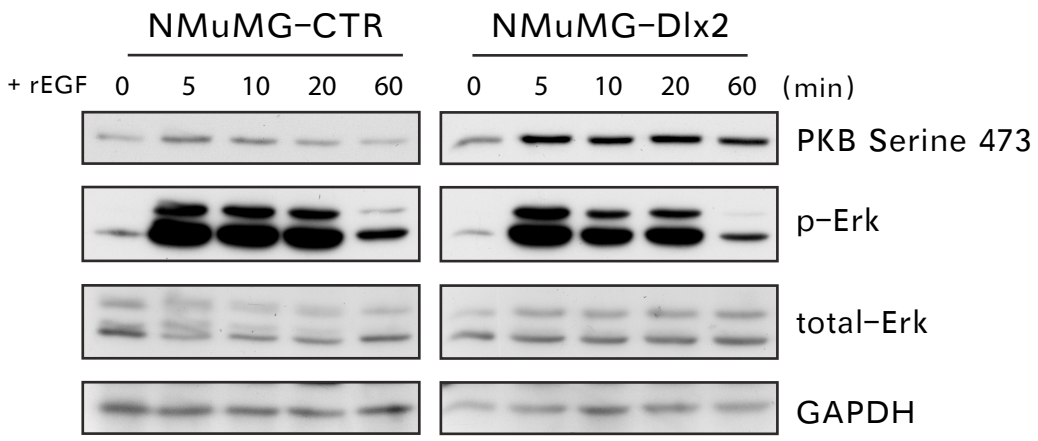


Figure S10. Dlx2 expression leads to increased EGFR activity. Immunoblotting analysis of Dlx2-expressing and control NMuMG cells treated with recombinant murine EGF (rEGF, 10ng/ml) for times indicated. Note that PKB Serine473 phosphorylation is increased and sustained in Dlx2-expressing NMuMG cells. Immunoblotting against GAPDH was used as a loading control.

3.2.6. Material and Methods

Reagents and antibodies

Recombinant human TGFβ (#240-B, Becton&Dickinson), recombinant murine EGF (#PMG8041, Invitrogen). **Antibodies:** E-cadherin for immunfluorescence (#13-1900, Zymed), for western blot (#610182, Transduction Laboratories), N-cadherin for immunfluorescence (#33-3900, Zymed) for western blot (#M142, Takara), NCAM (5B8, gift from U. Cavallaro), Vimentin (#V2258, Sigma-Aldrich), Vinculin (#V9131, Sigma-Aldrich), GAPDH (#ab9485, Abcam), TGFβRII (#sc-220, Santa Cruz), Smad4 (#sc-7154, Santa Cruz), Smad2 (#3103, Cell Signaling), pSmad2 (#3101, Cell Signaling), $p21^{CIP1}$(#556430, Pharmingen), c-Myc (# 06-340, Upstate Biotechnology), pPKB Threonine (#5106, Cell Signaling), pPKB Serine (#9271, Cell Signaling), pErk (#M-8159, Sigma-Aldrich), Erk (#M7927, Sigma-Aldrich), pFAK (#611806, Becton&Dickinson), HA.11 (#MMS-101R, Covance), anti-BrdU-FITC (# 347583, Becton&Dickinson). **Inhibitors:** MEK1/2 Inhibitor PD98,059 (#ALX-385-023, Alexis Biochemicals), TGFβRI inhibitor SB431542 hydrate (#S4317, Sigma-Aldrich), PI3K inhibitor ZSTK474 (#ALX-270-454, Alexis Biochemicals), PDGFR inhibitor Thyphostin AG1296 (#ALX-270-037, Alexis Biochemicals), PI3K Inhibitor AG1478 (#ALX-270-036, Alexis Biochemicals).

Cells and cell lines

A subclone of NMuMG cells (NMuMG/E9; hereafter NMuMG) expressing E-cadherin has been previously described (Maeda et al., 2005). MTdeltaEcad and MCF7-shEcad have been described (Lehembre et al., 2008). Cells were cultured in DMEM supplemented with glutamine, penicillin, streptomycin, and 10% FCS (Sigma). NMuMG-shSmad4 and NMuMG-shCont were obtained from P. ten Dijke (Leiden University Medical Center, The Netherlands; Deckers et al., 2006). TGFβ treatment of NMuMG cells was performed without serum deprivation, and TGFβ was replenished every 3 days (2 ng/ml). mDlx2 siRNA (ON-TARGET plus, SmartPool, L-043273-01-005, mouse Dlx2) was purchased from Dharmacon. mDlx2 shRNAs were purchased from Sigma-Aldrich. Transfections with LipofectAMINE RNAiMAX (Invitrogen) were performed according to the manufacturer's instructions. Total cell lysates, immunoblots, and immunofluorescence experiments were performed as

previously described (Lehembre et al., 2008). Depending on the species origin of antibodies, immunoblots were either probed sequentially or on multiple membranes. Adobe Photoshop has been used to excise the relevant portion of the immunoblots from the original scans of X-ray films exposed to chemiluminescence visualization of specific proteins, as indicated by black frames in the figures.

Production of lentivirus containing Dlx2 and shDlx2 short hairpin RNA.

Murine Dlx2 shRNAs (shDlx2 #1-3, TRCN0000070598-600) and control shRNA (shCTR, SHC002, Mission Non-Target shRNA Control Vector) were purchased from Sigma-Aldrich. Dlx2 (kindly provided by Prof. Dr. Peter Farlie) was tagged N-terminally with HA-tag and cloned into the lentiviral expression vector pWPXL. Lentiviral particles were produced by transfecting HEK293T cells with the lentiviral expression vectors in combination with the packing vector pR8.91 and the envelope encoding vector pVSV using Fugene HD. After two of virus production, lentivirus containing supernatants were harvested, filtered (0.45 μm) and added to target cells in presence of polybrene (8 ng/ml). Infections were performed twice a days for two days.

Quantitative RT-PCR

Total RNA was prepared using Trizol (Invitrogen), reverse transcribed with M-MLV reverse transcriptase RNAse (H-) (Promega, Wallisellen, Switzerland), and transcripts were quantified by PCR using SYBR-green PCR MasterMix (Applied Biosystems, Rotkreuz, Switzerland) and the primers indicated in Table S1. Human or mouse riboprotein L19 primers were used for normalization. PCR assays were performed in triplicates, and fold induction was calculated against control-treated cell lines using the comparative Ct method ($\Delta\Delta$ C_t). Following primer were used:

murine E-cadherin:

fwd: 5'-TTTACCCAGCCGGTCTTTGA-3', rev: 5'-TCCTGGAACAGCGCCTTCT-3';

murine N-Cadherin

fwd: 5'-CTGCCATGACTTTCTACGGAGA-3', 5'-CAATGACGTCCACCCTGTTCT-3';

murine Dlx2

fwd: 5'-GGCCTCACCCAAACTCAGGT-3', rev: 5'-GTATCTCGCCGCTTTTCCAC-3';
TGFβR2
fwd: 5'-GGCTCTGGTACTCTGGGAAA-3', rev: 5'-AATGGGGGCTCGTAATCCT-3';
murine RPL19:
fwd: 5'-ATCCGCAAGCCTGTGACTGT-3', rev: 5'-TCGGGCCAGGGTGTTTTT-3'

Reporter Assay

NMuMG cells were transfected with 200 ng reporter and 5 ng Renilla encoding plasmids using Lipofectamine 2000. After 2 days of transfection, cell were analyzed using the Dual-Luciferase Reporter Assay System (#E1960, Promega) and the Berthold Luminometer LB960. Measured luciferase values were normalized to internal Renilla control. Smad4 promotor construct kindly provided by Peter ten Dijke (Dennler et al., 1998). TGFβRII promotor reporter kindly provided by Seong-Jin Kim (Hahm et al., 1999). E-cadherin promotor reporter kindly provided by Kirstin Verschueren (van Grunsven et al., 2003)

Cell growth curves

$1x10^4$ cells were seeded in each well of 24-well plate and cell number was assessed by using a Neubauer counting chamber.

Migration assays

Cell migration was determined in a modified two-chamber migration assay (pore size: 8 μm; Falcon BD, Franklin Lakes, NJ). 10^5 cells were seeded in 1% fetal calf serum/DMEM (Sigma) in the upper chamber and the lower chamber was filled with 10% fetal calf serum/ DMEM. After 24h incubation at 37°C, cells in the upper chamber were carefully removed with a cotton swap and the cells that had traversed the membrane were fixed in 4% paraformaldehyde/HBS-Ca^{2+}, stained with crystal violet (0.5% in 20% methanol) and counted.

Proliferation assay (BrdU incorporation)

Cells were incubated with 10µM BrdU for 2h at 37C. Fixed in 70% ice-cold Ethanol. Treated with 2N HCL/0.5% Triton X-100 solution for 30 min at RT. Resuspended in 0.1 M Na2B4O7 pH 8.5 for 2min at RT. Washed with 0.5%Tween-20/1% BSA/PBS. Incubated with FITC labeled anti-BrdU AB (#347583, BD) for 30 min RT. Washed with 0.5%Tween-20/1% BSA/PBS. Resuspended and incubated in PBS with 5µg/ml PI for at least 1h RT. Filtered and analyzed by FACSCanto II using DIVA software.

Apoptosis assay (Annexin assay)

Cells were washed twice with cold PBS and resuspended in 1X Binding Buffer at a concentration of 1 x 10^5 cells/ml. 5 µl of Cy5 Annexin V was added to cells and incubated for 15 min at RT (25°C) in the dark. After 15 min incubation at RT, cells were analyzed on a FACSCanto II using DIVA software.

Subcutaneous injections

Cells were harvested by trypsinization and resuspended in PBS to a final concentration of 4x10^5 cells/200µl. 8 weeks old C57/Bl6 mice were injected in the flank with 200 µl for each cell line, and tumors were allowed to form for 14 days. Mice were sacrificed when the tumors reached a diameter of 1 cm. These experiments were repeated 2 times, using 5–8 mice per tumor group.

Collagen gel assay

Cells trypsinized from tissue culture plates were washed with PBS and counted. 1 x 10^5 cells in 333 µl media cell were mixed with 74 µl MEM, 37 µl Sodium Bicarbonate (# S8761, Sigma-Aldrich) and 555 µl PureCol (#5409, Inamed Biomaterials). The mixtures were distributed in chamber of 8-well CultureSlides (#354118, Becton&Dickinson) and allowed to solidify into gel for 1 hour at 37C. Subsequently, collagen gels were overlaid with complete

DMEM with or without TGFβ (2ng/ml). The medium with respective growth factors was changed every second day.

Histological analysis

The preparation of immunohistochemical analysis was performed as described previously (Perl et al., 1998). Stainings were evaluated on an AxioVert microscope and on a LSM 510 META confocal microscope (Zeiss, Oberkochen, Germany).

Statistical analysis

Statistical analysis and graphs were generated using the GraphPad Prism software (GraphPad Software Inc, San Diego, CA). All statistical analysis were done by unpaired, two-sided t-test. Normality testing was performed using the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie for p-values.

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6. PUBLICATIONS

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